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CHARACTERIZATION OF THE P. BREVIS POLYETHER NEUROTOXIN BINDING COMPONENT IN EXCITABLE MEMBRANES

FINAL REPORT

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The development of a functional model, and topographic picture of how and why sodium channels act in the ways in which they gate sodium ion flux is our goal. We are developing about 20 different natural toxin derivatives based on 7 divergent chemical modifications. Each type of derivative has a specific potential once synthesized. Photoaffinity probes, affinity columns, tritiated non-exchangeable toxins, and specific intermediates are in various stages of completion. These probes are being utilized to characterize the topographic relationship of sites 1, 2, and 5 associated with voltage-sensitive sodium channels. The brevetoxin binding site has already been localized on Domain IV of VSSC, and binds to an external hydrophobic peptide located between S5 and S6 of Domain IV. Antisodium channel RIA, tritiated brevetoxin photoaffinity binding, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis have all made substantial contributions to brevetoxin site localization.

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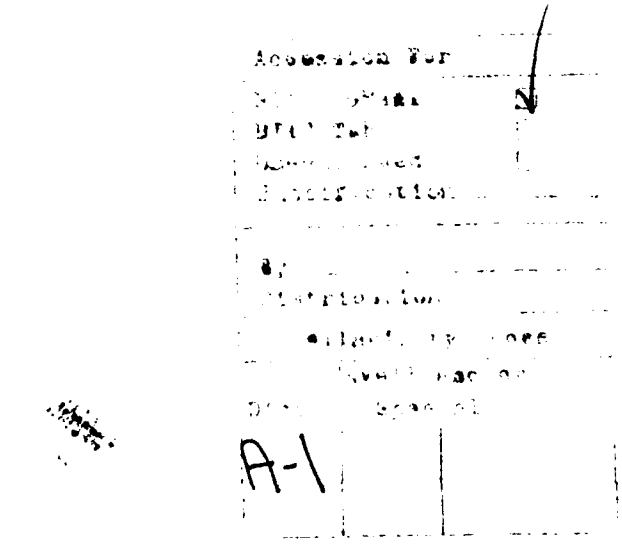

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

INTRODUCTION 5

Nature of the problem 5
Background 6
Purpose of Present Work 8

Photoaffinity Probes 8
Affinity Columns 9
Radioactive Toxins 9
Enzyme-Synthesized Tritiated Brevetoxins 9

BODY 9

Experimental Methods 9

Brevetoxin Site Characterization 9
Materials 9
Toxin Purification 10
Preparation of Synaptosomes 10
Binding Experiments and Covalent Modification 10
Multiple Brevetoxin Binding Site Experiments 10
Solubilization and Gel Filtration 11
SDS-PAGE 11
2-Mercaptoethanol Treatment 11
Neuraminidase Treatment 11
Ion Exchange and Affinity Chromatography 11
Sodium Channel Antibody 11
Measurement of Protein Concentration 11

Molecular Toxin Probes 11
Affinity Columns 11
Photoaffinity Probes 12
^{14}C-labeled Toxins 12
Tritiated Toxins 12

Molecular Modeling 13

Results and Discussion 14

Brevetoxin Binding Site Characterization Results 14
Brevetoxin Binding 14
Brevetoxin Photoaffinity Probe Preincubation 14
Brevetoxin:Saxitoxin Binding Ratio 14
Hill Plots of Brevetoxin Saturation Binding Data 15
Dependence of Brevetoxin Binding on Membrane Polarity 15
Photoaffinity Probe Stability 15
Irradiation Optimization 18
Binding Studies Using p-azidobenzoate brevetoxin 18

Low Affinity Binding 18
Gel Filtration 19
SDS-PAGE 19
Immunoprecipitation 20

Brevetoxin Binding Site Characterization Discussion 20
Brevetoxin Binding Experiments 20
Brevetoxin Photoaffinity Probe Preincubation 22
Brevetoxin:Saxitoxin Binding Ratio 22
Low Affinity Binding Site Characterization 22
Binding Site Class Function 23
Binding Site Purification 23

Brevetoxin Binding Site Modeling Results and Discussion 24

Toxin Derivatives and Deliverables 29
Affinity Columns 29
Photoaffinity Probes 30
Radioactive Toxins 30

Significant Negative Results 32

CONCLUSIONS 34

Implications of Completed Research 34
Future Work Recommendations 34

PUBLICATIONS AND MEETING ABSTRACTS ARISING FROM SUPPORT 35

PERSONNEL COVERED BY CONTRACT SUPPORT 37

GRADUATE DEGREES RESULTS FROM CONTRACT SUPPORT 37

REFERENCES 38

INTRODUCTION

Nature of the Problem

There are seven known sites associated with the voltage-sensitive sodium channel (VSSC) that bind low molecular weight natural toxins with high avidity and affinity (Table 1) (Wu and Narahashi, 1987; Strichartz et al, 1987; Ellis, 1985; Catterall and Risk, 1981, Poli et al, 1986; Sharkey et al, 1987; Catterall and Gainer, 1985, Lombet et al., 1987; Frelin et al, 1990). The unique nature of each of the binding sites was ascertained using radioactive toxin of one type, and attempting to "compete" away labeled toxin using an excess of unlabeled toxic material. Site 1 is known to bind saxitoxin, tetrodotoxin, and μ-conotoxin, and occupancy at this site blocks passage of sodium ions (Moczydlowski, et al., 1986).. Specific binding studies have been carried out using tritium-labeled saxitoxin and ^{125} I-labeled μ-conotoxin. Site 2 exhibits an affinity for batrachotoxin, the grayanotoxins, and the vertrum alkaloids. Binding at this site causes persistent activation of channels. Specific binding studies have used tritiated batrachotoxin α-benzoate. Site 3 binds sea anemone toxin and the α-scorpion toxin (*Leiurus quinquestratis*) and enhances persistent activation while inhibiting inactivation. Site 4 binds the ß-scorpion toxins (*Centroides suffusus suffusus*), causing repetitive firing and shifting activation voltages. Site 5 binds the trans-fused polyether dinoflagellate brevetoxins and ciguatoxin. Binding at site 5 leads to a shift in activation potential to more negative values, leading to persistent acitvation at normal membrane potential. Ciguatoxin is the only toxin that has been shown to inhibit the binding of tritiated brevetoxin to site 5. Based on inhibition constant data for ciguatoxin, it must bind with higher affinity than do the brevetoxins. Sites 6 and 7 bind the pyrethroids and pumiliotoxin B; site 6 being allosterically-linked to the brevetoxin site (Lombet et al., 1987; Frelin et al., 1990; Bidard et al., 1984) and site 7 also is linked to site 5 allosterically (Gusovsky et al., 1988).

TABLE 1. RECEPTOR BINDING SITES ASSOCIATED WITH VSSC [1]

Receptor Site	Ligand	Physiological Effect
1	Tetrodotoxin, saxitoxin, μ-conotoxins	Inhibit ion conductance
2	Batrachotoxin, veratrum alkaloids, grayanotoxins	Persistent activation
3	α-Scorpion toxin, sea anemone toxin	Inhibit inactivation
4	ß-Scorpion toxin	Shift activation
5	Brevetoxins, ciguatoxin	Shift activation, inhibit inactivation
6	Pyrethroids	Shift activation, inhibit inactivation
7	Pumiliotoxin B	Inhibit inactivation

[1] from reference (Baden, 1989).

Highly specific ligands like the natural toxins, have been indispensible in the description of the topographic and functional character of the VSSC. These specific ligands have aided in determination of sodium channel number, in localization of sodium channels in tissues, in developing working hypotheses on

the allosteric functional properties of the channel, in identification and purification of individual protein components which make up the channels, and in development of new therapeutics which mimic the action of these drugs (Catterall, 1985).

These tasks can be successfully undertaken for several reasons: (i) most of the natural toxins are available in purified forms and sufficient quantities for micro-organic manipulation; (ii) each toxin type interacts with their channel binding site with affinities in the nM to pM concentration ranges; (iii) toxin structures may be manipulated to provide derivatives which aid in developing structure-activity relationships; (iv) toxin structure may be modified in conservative ways which produce radioactive, photoaffinity-linked, and solid-support immobilized derivatives. We have chosen to direct our attention to the production and study of ligand derivatives specific for VSSC sites 1, 2, and 5---those defined sites for which the ligands are non-proteinaceous natural toxins. These toxin probes shall be utilized to study the interaction with Site 1 (saxitoxin and derivatives, tetrodotoxin), site 2 (batrachotoxin, veratridine, aconitine, and the grayanotoxins), or site 5 (brevetoxins) on sodium channels.

The following derivatives have been proposed for synthesis:

(a) solid-support linked brevetoxin, batrachotoxin, veratridine, and saxitoxin columns;
(b) photoaffinity-linked derivatives of tritium-labeled brevetoxins;
(c) non-toxic brevetoxin photoaffinity derivatives;
(d) tritium-labeled tetrodotoxin and saxitoxin, batrachotoxin, and veratridine and their corresponding photoaffinity labels;
(e) high specific activity ^{125} Iodine-labeled photoaffinity-coupled saxitoxin, batrachotoxin, veratridine, and brevetoxin;
(f) enzyme-synthesized brevetoxin (PbTx-3) with theoretical specific activities approaching 50 Ci/mmole; and
(g) ^{14} Carbon brevetoxin (PbTx-3).

As described later, items (a), (b), (c), (g) and portions of (d), and (e) are complete. The remainder of (d) and (e), and an alternative method for obtaining (f) are progressing.

The derivatives continue to be utilized to:

(a) determine the spatial relationship of Site 1, Site 2, and Site 5 in rat brain synaptosomes;
(b) isolate and purify Site 1, Site 2, and Site 5 toxin binding components from rat brain synaptosomes;
(c) characterize specific binding receptors in other model systems like neuroblastoma cell lines;
(d) investigate the characteristics of brevetoxin receptors in pulmonary cell lines;
(e) investigate brevetoxin metabolism in an hepatocyte tissue culture model system.

Background

Sodium channels isolated from rat brain consist of three separate and separable protein subunits, the α-subunit and ß1- and ß2-subunits, a 1:1:1 stoichiometry composing the channel (Catterall, 1989). The α-subunit is a glycoprotein of approximately 260 kDa molecular weight is a transmembrane protein which binds neurotoxins at specific positions on its topographic surface. The two ß- subunits are smaller molecular weight peptides (ca. 30 kDa each) and are integral membrane subunit glycoproteins. A schematic representation of the voltage-sensitive sodium channel is given in figure 1.

Using the primary structure data (Noda et al, 1986; Tanabe et al., 1987), Catterall (1989) developed a model for how the α-subunit inserts itself into the membrane of excitable cell types. Each α-subunit consists of 4 homologous domains; each homologous domain being composed of 6 transmembrane peptide sequences (figure 2). Using site-directed antibodies, Catterall has shown that specific sites are phosphorylated during activation and inactivation, and using ^{125} I-labeled α-scorpion toxin in conjunction with protein A-sepharose precipitation of antibody-toxin associations (Catterall, 1989). Within each domain, the 6 transmembrane

sequences also appear to be conserved (Noda et al., 1986), with the highly positively charged S4 regions (denoted by "+" in the figure) being most highly conserved (Tanabe et al., 1987). These S4 domains have been postulated to completely transverse the membrane, and all 4 S4 regions in concert contribute to the ion shuttling ability of the α-subunit (Catterall, 1989).

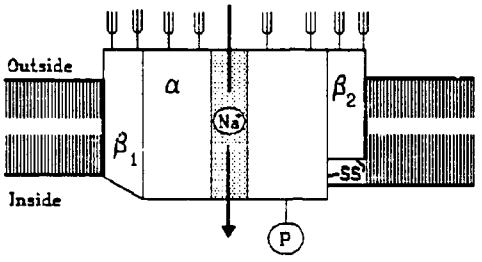

Figure 1. Subunit structure of sodium channel. Disulfide bonds are indicated by (S-S), phosphorylation sites by (P), and glcosylation sites by pitchforks. Removal of the ß1-subunit causes loss of specific saxitoxin binding (Catterall, 1985), and solubilization reduces specific binding at sites 2 and 3 (Messner et al., 1986). The primary structure of the α-subunit has been determined (Noda et al., 1986) and the subunit has been cloned Tanabe et al., 1987). Diagram is from Catterall (1989).

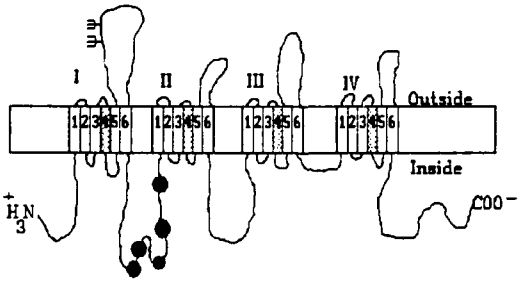

Figure 2. Catterall's functional map of the α-subunit of voltage-sensitive sodium channel. The α-subunit of sodium channel (modified from Lombet et al., 1987) is shown with phosphorylation sites, with sites of specific interaction with antibodies, glycosylation sites, and scorpion toxin binding site. The S4 region, shown with shading is thought to be responsible for voltage-sensitive ion transport, perhaps by the "sliding helix" model (Catterall, 1989).

Purpose of the Present Work

The development of a functional model, and a topographic picture of how and why α-subunit acts in the way in which it does, is central to the goals of this contract work. Once functional maps have been developed, the molecular mechanism and mechanics of VSSC action can be explained. The work we are undertaking involves the development and use of no fewer than 20 different natural toxin derivatives based on 7 divergent chemical modifications. The principal toxins we have worked with are the brevetoxins, saxitoxin, tetrodotoxin, veratridine, and aconitine. Our original proposal included batrachotoxin, but insufficient material could be obtained to carry out the work. The latter two site 2 toxins were developed instead. Each type of derivative has a specific potential use once synthesized. The chemical methods by which each toxin type is derivatized is different, but the "probes" developed are similar. Most recently, the use of molecular modeling by computer using the software MacroModel developed by Clark Still, has provided us with additional quantitative information which has led to the development of a molecular working model for polyether marine toxin and sodium channels.

Photoaffinity Probes.

As already described briefly in the Introduction, and amply reported in the literature, physiological and pharmacological aspects of toxin interaction with VSSC are well documented. The critical element of photoaffinity probes is their ability to form covalent attachments with the specific receptors associated with the pharmacological action. The inherent usefulness of radioactive photoaffinity probes is that the label does not dissociate under conditions of receptor denaturation, making it possible to experimentally purify the receptor and utilize the radioactivity as a specific marker for the receptor. At the molecular level, peptides to which natural toxin ligands attach can be identified by SDS polyacrylamide gel electrophoresis, if the labeled toxin is attached covalently and is "hot" enough radiochemically. Molecular weights can be determined, and specific enzymatic cleavage of photoaffinity linked materials can be undertaken. Ultimately, a purified low molecular weight peptide can be sequenced to determine the precise amino acid to which the toxin binds.

A second useful technique is to use specific anti-sodium channel antibodies to detect peptide which contain labeled photoaffinity probes attached. Or, anti-toxin antibodies can be used in Western blots of polyacrylamide gels to ascertain peptides which have unlabeled toxin photoprobes attached. Additional photoaffinity labeled toxins can be utilized to ascertain the identical or different nature of peptides which bind two types of toxins. Similarly, if both low and high affinity sites are postulated, competitive toxin ligands can be used to displace photoaffinity probes from high or low affinity sites, thereby labeling only one of two or more receptor sub-types. Certainly, several different photoaffinity linked toxin probes would be useful in determining polypeptide distance relationships, multiplicity, and allostery.

Isolation of larger quantities of specific toxin receptor requires some type of non-dissociable label, and photoaffinity probes satisfy this requirement exquisitely. Since the label in the bulk solution is rapidly inactivated, the observed specificity should be greater than that obtained with toxin alone under the same conditions. This means that label activated in the bulk solution cannot contribute to the site-specific labeling. If the nitrenes generated react as rapidly with adjacent solvent molecules as expected, labeling should be highly specific, even with excess label. Thus, photoaffinity labeling of low, as well as high, affinity sites should be possible. Also, labeling of hydrophobic sites should be possible, since nitrene produced upon photolysis can react with carbon-hydrogen bonds as well as nucleophilic functions.

For different uses, we described the synthesis of two different types of photoaffinity probe (Baden, 1990). The first is of small size (relative to the toxins themselves) and is tritiated in a non-exchangeable form. The second is a more elaborate probe, employable for carboxylic acid, amine, and hydroxyl linkage, and is iodinatable. The latter type will be most applicable to autoradiography and rapid non-quantitative measurement. The former will likely more specifically probe the high affinity binding sites in close proximity to the active sites of receptors. For purposes of this contract, we have concentrated on synthesis of the smaller, more straightforward photoaffinity probes, beginning with their unlabeled counterparts and determining the chemical characteristics of the derivatives. All unlabeled photoaffinity probe synthesis was successful.

Affinity Columns.

In theory, affinity chromatography offers the most straightforward means of purifying natural toxin binding sites because it relies on well-defined biospecific binding properties of receptors to accomplish purification. With specific toxin-receptors dissociation constants in the nM to pM concentration ranges, time constants for purification range from several minutes to a couple of days. Problems which arise during affinity chromatography can include such diverse encounters as: (i) receptor being irreversibly bound due to very high affinity (fortunately not a usual case); (ii) loss of receptor activity upon solubilization prior to affinity separation; (iii) the low percentage of sought receptor in the entire mileau of material applied to the column leading to "masking" of ligand on the column; and (iv) coupled ligand which loses its ability to reversibly bind receptor. By our accounts, each of the proposed affinity columns has been synthesized to produce covalently-linked toxin to Sepharose solid support.

Radioactive Toxins.

This particular area is probably the most difficult to approach for several reasons: (i) the toxins produced in radioactive form should be as close to the original toxin structure as possible. This generally involves oxidation and reduction reactions to introduce tritium into the molecules; (ii) the tritium introduced should be non-exchangeable. This is to say, the tritium should not exchange for hydrogen with either solvent or any biological material. Many tritiated toxins presently available suffer from back-exchange reactions; (iii) the tritium introduced should be of high specific activity, preferably 20-100 Ci/mmole. Only three companies make isotopes for labeling at this specific activity, and the availability is limited and difficult to schedule; (iv) the labeled toxin should be stable in auqeous solution for binding studies.

For metabolic studies, brevetoxins have been biosynthesized in a uniformly labeled form, and of ^{14}C composition. This material was produced in cultures using radioactive bicarbonate and fixing carbon dioxide for incorporation. Both saxitoxin and okadaic acid have been produced in ^{14}C uniform label form, as well.

Enzyme Synthesized Tritiated Brevetoxins.

Based on our past knowledge about the metabolic interconversion of brevetoxin PbTx-2 (aldehyde) to brevetoxin PbTx-3 (alcohol), catalyzed by alcohol dehydrogenase, we anticipated being able to tritiated brevetoxins by an NAD^3 H reaction using yeast alcohol dehydrogenase. The expected specific activity was very high relative to chemical reductions, and specific. Oxidation of PbTx-2 to the carboxylic acid followed by enzymatic reduction was to yield a toxin in which two tritiums are added per molecule, thereby doubling the specific activity. Reduction of PbTx-3 to PbTx-9 (reduction of the α-methylene) would add another two tritiums if done in tritiated solvent, leading to a toxin with a conservative specific activity of 50 Ci/mmole. The low specific activity of borotritiide we have repeatedly obtained precluded high specific activity synthesis. As an alternative, we are producing O-methyl PbTx-2 by a methylation reaction using C^3H_3I (60Ci/mmole). A realized advantage of this reaction is that we can produce methylated derivatives of PbTx-1, PbTx-2, PbTx-3, PbTx-5, PbTx-7, PbTx-9 and PbTx-10 and hotter photoaffinity probe necessary for peptide localization using the new derivative.

BODY

Experimental Methods

Brevetoxin Site Characterization

Materials - Sephacryl S-300, Sephadex A-25, and wheat germ agglutinin-Sepharose were obtained from Pharmacia and Soluene 350 tissue solubilizer was obtained from Packard Instrument Co. All electrophoresis chemicals and 2-mercaptoethanol were purchased from Fisher Scientific Co. Organic counting scintillant (OCS), standard high molecular weight rainbow protein markers, and sodium borotritiide were from Amersham Corp. Aquasol was purchased from New England Nuclear. All remaining chemicals were reagent grade or better and were purchased from Sigma. All enzymes were used without further purification.

Toxin purification - Brevetoxins PbTx-2 and PbTx-3 (Fig. 3) were extracted from stationary phase laboratory cultures of *Pychodiscus brevis* by a combination of chloroform/methanol extraction, thin layer chromatography and reverse phase HPLC (Baden et al., 1981; Poli et al., 1986). Both [^{3}H]PbTx-3 and unlabeled PbTx-3 were synthesized by reduction of the PbTx-2 aldehyde using sodium borotritiide or sodium borohydride, respectively (Poli et al., 1986). Specific activity of the radioactive preparation was determined by HPLC mass quantification at 215 nm against toxin standards and scintillation spectrometry against [^{3}H]methanol standards. Tritiated PbTx-3 used for synthesis of the photoaffinity probe had a specific radioactivity in the range of 10-13 Ci/mmol.

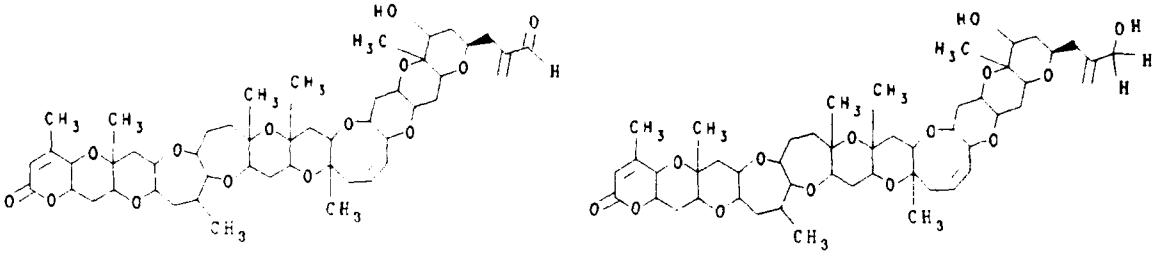

Figure 3. Structures of brevetoxin PbTx-2 (left) and brevetoxin PbTx-3, illustrating the trans-fused polyether structure and the differences betwenn the two toxins on the side chain of ring K.

Competitor toxins and photoaffinity-linked toxin derivatives were weighed on a Cahn gram electrobalance and dissolved in ethanol, resulting in stock solutions of 0.5 - 1.0 mM. Competitor toxin concentrations were verified by HPLC against defined standards.

Preparation of synaptosomes - The method described by Dodd et al., (1981) was used for preparation of synaptosomes from the brains of Sprague-Dawley rats. Synaptosomes were found to retain brevetoxin binding activity for several months when stored at -70°C.

Binding experiments and covalent modification - Binding of [^{3}H]PbTx-3 p-azidobenzoate (hereafter designated as [^{3}H]PbTx3-Pho) was determined using a rapid centrifugation technique as described by Poli et al. (1986). Synaptosomes, at a final protein concentration of 100 µg/ml, were added to reaction vials containing [^{3}H]PbTx3-Pho (total binding). Nonspecific binding was assayed by addition of 10 µM unlabeled PbTx3-Pho. The entire experiment was carried out in subdued room light. After a one hour incubation of [^{3}H]PbTx-3 with synaptosomes at 0°C, samples were washed three times by dilution and centrifugation with binding medium containing 1% BSA. Samples were transferred to petri dishes and exposed to UV irradiation (Model UVG-11, UVG Inc., 254 nm maximum, 18 W, at a distance of 1 cm) for 5 min with gentle agitation. Data from Rosenthal analysis were analyzed using LIGAND (Munson and Robard, 1980).

Multiple brevetoxin binding site experiments - With the exception of assays which employed depolarized synaptosomes, all binding experiments were performed in binding medium consisting of 50 mM HEPES (pH 7.4), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magnesium sulfate, 5.4 mM potassium chloride, 1 mg/ml bovine serum albumin (BSA), and 0.01% Emulphor EL-620, a nonionic detergent used as an emulsifier. The latter was required in order to solubilize the high concentrations of unlabeled PbTx-3 used in the measurement of nonspecific binding. For measurement of brevetoxin binding to depolarized synaptosomes, binding medium consisted of 50 mM HEPES (pH 7.4), 135 mM potassium chloride, 5.5 mM glucose, 0.8 mM magnesium sulfate, 1 mg/ml BSA, and 0.01% Emulphor. Step-wise depolarization was achieved by varying the potassium chloride to choline chloride ratio in order to obtain different degrees of membrane depolarization.

In order to investigate the possibility of multiple binding site classes, saturation binding experiments employed [^{3}H] PbTx-3 concentrations from 0.19 - 175 nM and [^{3}H] p-azidobenzoate brevetoxin concentrations from 0.8 - 100 nM. Radiolabeled saxitoxin binding as well as binding of [^{3}H] PbTx-3 to intact, 135 mM KCl depolarized, and osmotically lysed synaptosomes were measured at radiolabeled toxin concentrations ranging from 0.78 - 25 nM. Step-wise KCl depolarization was measured at 10 nM [^{3}H] PbTx-3. Binding data were

subjected to Scatchard analysis as well as curve fitting using LIGAND (Munson and Rodbard, 1980). The statistical significance of data fit to models was determined by performing an f test on regressions computed by LIGAND.

Solubilization and gel filtration - Solubilization of sodium channel covalently-linked to the brevetoxin photoaffinity probe (hereafter designated as PbTx-Pho NaCh) using Triton X-100 and gel filtration using a Sephacryl S-300 column were performed as described by Hartshorne et al.(1980).

SDS-PAGE - Irradiated samples were centrifuged for 2.5 min at 15,000 x g and pellets were prepared for SDS-PAGE by resuspension in non-reducing sample buffer (Laemmli, 1970) and incubated at 100°C for 2 min. Samples were electrophoresed on 1.5 mm thick SDS acrylamide gels prepared by the method of Blattler et al. (1972).

2-mercaptoethanol treatment - One ml of solubilized synaptosomes (both total and nonspecific preparations were treated in an identical manner) was incubated for 4 min at 100°C with 15 mM 2-mercaptoethanol. Iodoacetamide in 0.1 M Tris-HCl (pH 8) was added to yield a final concentration of 45 mM and the mixture was incubated at 100°C for 1 min (Sharkey et al., 1984). After cooling to 4°C, this solution was loaded onto the Sephacryl column.

Neuraminidase treatment - Control enzyme activity was assayed as described by Warren (1959). Neuraminidase treatment followed the protocol described by Messner and Catterall (1985). Both total and nonspecific column eluents (2 ml aliquots) of the putative α-subunit were recovered from the 2-mercaptoethanol treatment and incubated with 0.1 unit of neuraminidase at 37°C for 5 hours. A 0.5 ml aliquot of each digest was added to the Sephacryl column.

Ion exchange and affinity chromatography - Ion exchange chromatography using DEAE Sephadex A-25 and wheat germ agglutinin-Sepharose chromatography were performed as previously described (Hartshorne and Catterall, 1984) except that 1M KCl was used to elute the brevetoxin-linked membrane protein from the ion exchange column instead of 0.2M.

Sodium channel antibody - A synthetic peptide to residues 427 to 445 of the sodium channel α subunit (designated SP1) was prepared using the method described by Gordon et al. (1984). A standard glutaraldehyde linkage of the purified peptide to BSA was performed using the method of Orth (1979). The antibody to SP1 was prepared using the immunization protocol described by Gordon et al. (1987).

Measurement of protein concentration - Protein concentration was measured spectrophotometrically at 540 nm in ELISA plates using the bicinchonic acid assay (BCA; Pierce Chemical Co.) or at 595 nm using the Bradford method (Bradford, 1976; Bio-Rad Laboratories). BSA dissolved in mobile phase was used as a standard in both assays.

Molecular Toxin Probes

Affinity Columns - Brevetoxin PbTx-3 affinity columns were constructed using aminohexyl-Sepharose as solid support (1-6 μmole amino function/mL solid support, Pharmacia). Coupling PbTx-3-succinate was achieved using standard carbodiimide reactions with a small amount (about 1 μCi) of tritiated PbTx-3 added as tracer for assessment of coupling stoichiometry. PbTx-3 succinate was synthesized using succinic anhydride as previously described , purifying the derivative by thin-layer chromatography, and spraying a portion of the developed plate with bromocresol purple to detect the succinic acid-toxin derivative (Baden et al., 1984). On a Buchner funnel, 5 g dry aminohexyl-Sepharose was washed with 3 x 50 mL of distilled water, followed by three 50 mL rinses of 50% pyridine. Five mL (5-30 μmole amino functional group equivalents) of the resulting slurry was placed in a 50 mL beaker on a rotary shaker (30 oscillations/min) and 300 μmmole EDC (1-ethyl-3(-dimethylaminopropyl)carbodiimide) was added in 0.5 mL 50% pyridine. The mixture was allowed to swirl for 2 hr at room temperature, after which time 9.9 mg PbTx-3-succinate (10 μmole toxin equivalents) was added in 1 mL 50% pyridine. The reaction mixture was swirled at room temperature overnight, and the following morning was packed in a 25 mL glass column, rinsed sequentially with 1 column volume each of 50% pyridine, distilled water, and finally PBS pH 7.4 without sodium azide (Fig 4). As required for experiments, portions of the lyophilized, purified IgG from the Protein G eluate were rehydrated and purified using the brevetoxin affinity column. The column was pre-washed with 10 mM Tris buffer pH 7.5, followed by 10 bed volumes of

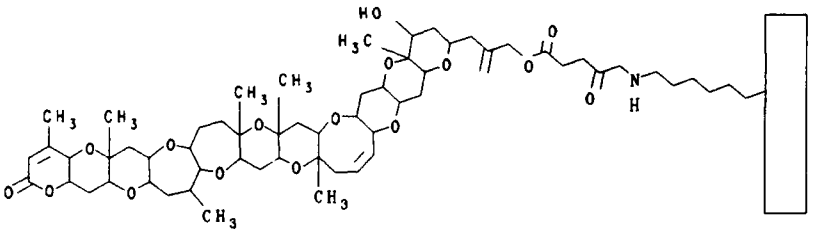

Figure 4. Brevetoxin PbTx-3 affinity column employing aminohexyl Sepharose.

100 mM glycine (pH 2.5), 10 volumes of both 10 mM Tris (pH 8.8) and 10 mM Tris pH 7.5. The pH of the column was never allowed above pH 9.0, where brevetoxins are unstable (Bunner *et al.*, 1990).

Brevetoxin PbTx-3, decarbamoyl saxitoxin, veratridine, and aconitine were also coupled to carboxyhexyl Sepharose using the following procedure. Carboxyhexyl Sepharose (0.5 g) was mixed with 0.162 g carbonyldiimidazole in 10 mL dry benzene, and was allowed to react with stirring at room temperature overnight. For each toxin alcohol (all toxin ligands have exposed hydroxyl functions), 20% of the carbonyldiimidazole-carboxyhexyl Sepharose complex was added and heated at 70°C overnight with gentle swirling on an oscillatory bench (DO NOT STIR WITH STIR BAR). Following reaction, conjugates were filtered and washed with: methanol->ethanol->water (saxitoxin), water->acetone (brevetoxin), chloroform->water (acontine), and acetone-> water (veratridine). Each washed conjugate was resuspended in water three times, filtered, and lyophilized. Unbound toxin (in the combined washes) was weighed and the difference between that added and that recovered was used to estimate bound toxin.

Photoaffinity Probes - The method described by Rao and Venkataraman (1939) was used to synthesize p-azidobenzoic acid. Coupling to toxin was carried out in the dark. Carbonyldiimidazole (10 µmoles) was mixed with an equimolar amount of p-azidobenzoic acid in 10 ml of dry benzene and was allowed to react for 3 hr in the dark at room temperature. One micromole of reacted p-azidobenzoic acid (1 ml) was added to 1 µmole dry toxin (brevetoxin, decarbamoyl saxitoxin, veratridine or aconitine), sealed in a thick walled glass vial, and heated in a mineral oil bath for 24 hr at 70°C. The reaction product was dried under a stream of nitrogen, resuspended in acetone and chromatographed on silica gel in petroleum ether:acetone (70:30). The desired fraction was visualized by exposing a small portion of the plate to UV light. The photoaffinity-labeled brevetoxin appeared as a colorless UV-fluorescent band with other minor products remaining at the plate origin.

For radioactive toxin conjugates (brevetoxin alone thus far), one cm fractions of the developed and irradiated TLC plate were scraped and assayed for ^{3}H by scintillation spectroscopy. The remaining unirradiated photoaffinity probe was scraped from the silica gel chromatography plate, eluted in acetone, flash-evaporated and purified by HPLC at 215 nm in 85% isocratic aqueous methanol. Subsequent NMR spectroscopy of the purified photoaffinity probe confirmed its identity (not shown).

^{14}C-labeled Toxins - Brevetoxins, saxitoxin, and okadaic acid were uniformly labeled with radioactive carbon using photosynthetic incorporation of $^{14}CO_2$ into logarithmic phase cultures of *Ptychodiscus brevis*, *Gymnodinium catenatum*, and *Prorocentrum lima*. In the case of the brevetoxins, each of the major toxins is anticipated in routine yield, saxitoxin and at least 5 derivatives were to be obtained, and both okadaic acid and DTX-1 were anticipated. Cultures (2 Liters of each, 1 x 10^4 cells/mL) were placed each in separate 8 liter carboys (6 liters of free air volume above cultures in carboys), fitted with a silicone stopper containing a 10 mL syringe with needle for introduction of radioactive solution. Each sealed container received 0.33 mCi $Na^{14}CO_3$ at three day intervals for 9 days. On the 11 day after initiation, cells were harvested and toxin purified using routine procedures already described.

Tritiated Toxins - Tritiated *Ptychodiscus brevis* toxin 3 (PbTx-3; Fig. 5) was prepared at a specific activity of 12-15 Ci/mmole by reductive tritiation of PbTx-2 (Fig. 3 left) using sodium borotritiide (Baden, 1989; Poli et al, 1986). This resulted in covalent incorporation of tritium into brevetoxin PbTx-3 at the C-42 position. Specific activity was calculated using HPLC against toxin standards and liquid scintillation counting standardized

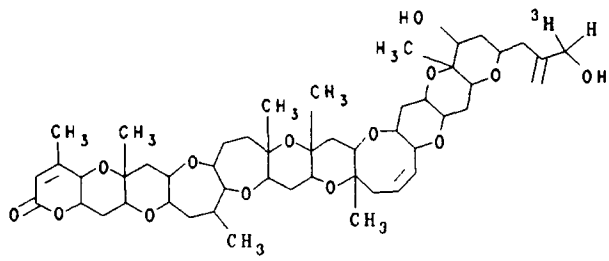

Figure 5. Tritium-labeled PbTx-3 produced using sodium borotritiide.

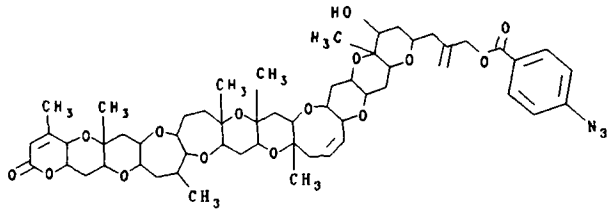

Figure 6. photoaffinity probe p-azidobenzoic acid linked to brevetoxin PbTx-3.

to a tritium quench curve. Brevetoxin p-azidobenzoate photoaffinity probe (Fig. 6) was synthesized as described in Trainer et al., (1991) and stored in brown borosilicate sample vials. Tritiated saxitoxin with a specific activity of 25 Ci/mmole was purchased from New England Nuclear.

Molecular Modeling of Brevetoxin

The most efficient method for randomly sampling conformational space makes use of internal coordinate Monte-Carlo subroutine techniques (Chang et al., 1989). This approach has recently been compared to other computational algorithms to locate the 256 low energy minima of cycloheptadecane (16 rotatable bonds), and was found to be far superior to its competitors (Saunders et al., 1990). The Monte Carlo method is incorporated into the molecular modeling software Macromodel, developed by Prof. Clark Still at Columbia University. Because the Monte Carlo method has not been explicitly tested on unsymmetric structures like these ladder toxins, we have approached the problem of multiple ring flexibility with caution.

For PbTx-2 type toxins, there are only two areas where the molecule might flex: the two 7-membered rings D and E, and the 8-membered ring H. All other rings in the molecule are held rigid by virtue of their trans-fused decalin-like geometries. Thus, these hinge points were modeled independently of one another using Monte Carlo methods on smaller models. The starting structures were randomly varied using the standard variables of Batchmin (the Macromodel batch-mode program). All bonds not specifically locked by their inclusion in a rigid ring were varied. Thus, for example, the ring fusion between rings C and D of PbTx-2 is rigid whereas the ring fusion between rings D and E is not. All work was carried out using MacroModel version V3.1X and associated BatchMin V 3.1 on a Silicon Graphics Personal Iris UNIX computer.

Results and Discussion

Brevetoxin Binding Site Characterization Results

Brevetoxin Binding - Experiments in the present study used a greater range of [^{3}H] PbTx-3 concentrations (0.19 - 175 nM) than the 0 - 25 nM range reported previously (Poli, 1986). Biphasic Scatchard plots were obtained (fig 7), suggesting the presence of multiple binding site classes.

Data analysis using LIGAND demonstrates that a two binding site class model gives a better statistical fit ($p < 0.001$) than does a one class model. The best fit estimate by LIGAND for a two-site model yields a Kd of 1.89 nM and a Bmax of 1.94 pmol PbTx-3/mg protein for the HA/LC binding site class and a Kd of 210 nM and a Bmax of 111 pmol PbTx-3/mg protein for the LA/HC binding site class. LIGAND was unable to fit the data to a model possessing more than two sites.

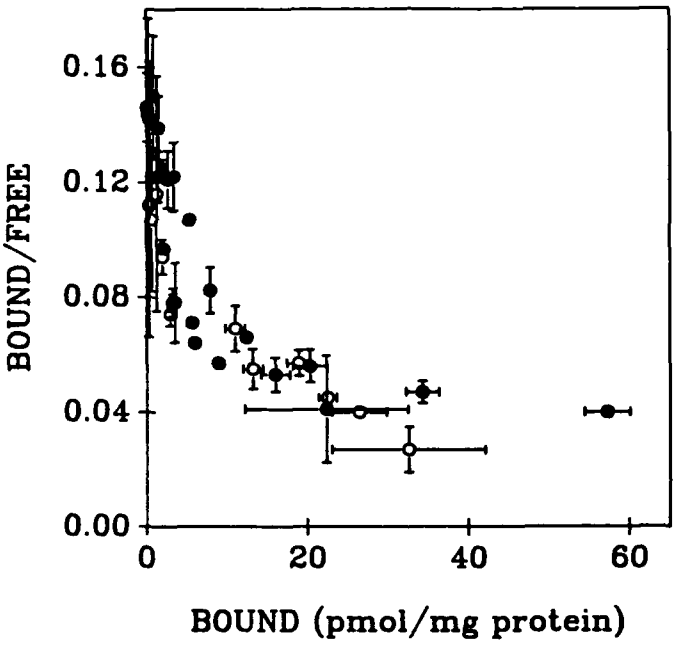

Figure 7. Scatchard analysis of tritiated brevetoxin saturation binding. Synaptosomes were incubated with 0.19-175 nM radioactive toxin. Open circles represent binding in the presence of 1 μM tetrodotoxin. Data points were pooled from three independent experiments and each point represents the mean of triplicate determinations. Error bars span ranges.

Brevetoxin Photoaffinity Probe Preincubation. Saturation binding experiments were performed in which synaptosomes were preincubated with 5 nM unlabeled brevetoxin photoaffinity probe and irradiated. Control experiments in the absence of photoaffinity probe resulted in a biphasic Scatchard plot (Fig. 8a) similar to the 2-site fit for underivatized brevetoxin observed in Fig. 7. LIGAND analysis indicates a better fit to a two site model compared to a one site model ($p < 0.01$). Best estimates by LIGAND yielded Kd values of 0.21 nM and 50.7 nM, and Bmax values of 2.12 pmoles/mg and 91.5 pmoles/mg protein for the HA/LC and LA/HC site classes, respectively. Preincubation with photoaffinity probe resulted in a linear Scatchard plot with a Kd value of 62 nM and a Bmax of 84 pmol/mg protein (Fig. 8b). The regression line was not statistically different from that of the derived LA/HC binding site regression observed in control Scatchard analyses ($p < 0.01$; Fig. 8a).

Brevetoxin:Saxitoxin Binding Ratio. Parallel saturation binding experiments compared the binding of [^{3}H] PbTx-3 and [^{3}H] STX to rat brain synaptosomes. The HA/LC brevetoxin binding sites and STX binding sites

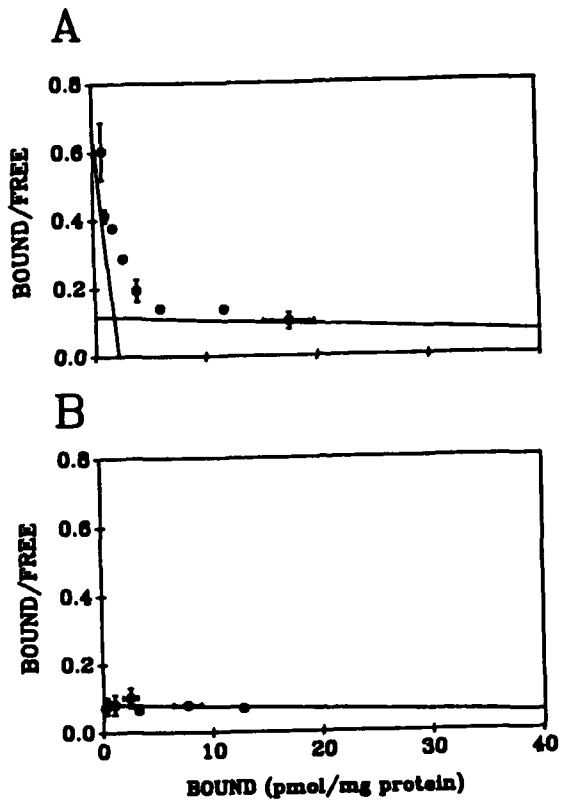

Figure 8. Scatchard analysis of tririated brevetoxin saturation binding to synaptosomes preincubated in the absence (A) and presence (B) of 5 nM unlabeled brevetoxin photoaffinity probe. In (B), following a 1 hr incubation with 5 nM p-azidobenzoic acid brevetoxin, the solution was irradiated at a distance of 1 cm with a hand-held uv lamp. Points represent the mean of triplicates within three identical experiments. Error bars span the range in each case.

are present in equal numbers, since the Bmax values of saxitoxin (1.72 $\pm$ 0.72 pmol/mg protein; n=3) and the LIGAND-derived brevetoxin HA/LC binding site class (1.94 $\pm$ 0.98 pmol/mg protein; n=3) are not statistically different ($p < 0.001$; Fig. 9).

Hill Plots of Brevetoxin Saturation Binding Data. In order to test the possibility and extent of allosteric interaction between the multiple classes of brevetoxin binding sites, Hill plots were generated from specific binding data (Fig. 10). The calculated Hill coefficient (1.00 $\pm$ 0.02; n=4) illustrates the lack of cooperativity between the HA/LC and LA/HC brevetoxin binding sites.

Dependence of Brevetoxin Binding on Membrane Polarity. We examined the possibility that membrane depolarization was the cause of changes in binding affinity at higher brevetoxin concentrations. At fixed 10 nM [^{3}H] PbTx-3 concentration, step-wise depolarization experiments reveal no significant change in apparent specific binding with increasing potassium chloride concentration (Fig. 11). The use of choline chloride buffer eliminated any membrane depolarization due to transmembrane ion flux, indicating that the synaptosomes were not already depolarized by the brevetoxin at low KCl concentrations. In addition, saturation binding experiments were performed using intact, osmotically lysed, and 135 mM KCl-depolarized synaptosomes (Fig. 12). There was no significant difference in Kd or Bmax values of HA/LC [^{3}H] PbTx-3 binding between intact, lysed, and depolarized synaptosomes ($p < 0.01$). In addition, no difference was observed in saturation binding experiments performed in the presence and absence of tetrodotoxin (Fig. 7). These results indicate that the binding of PbTx-3 to its specific site is essentially membrane-potential independent.

Photoaffinity probe stability - Analysis of the p-azidobenzoyl photoaffinity probe by reverse phase HPLC

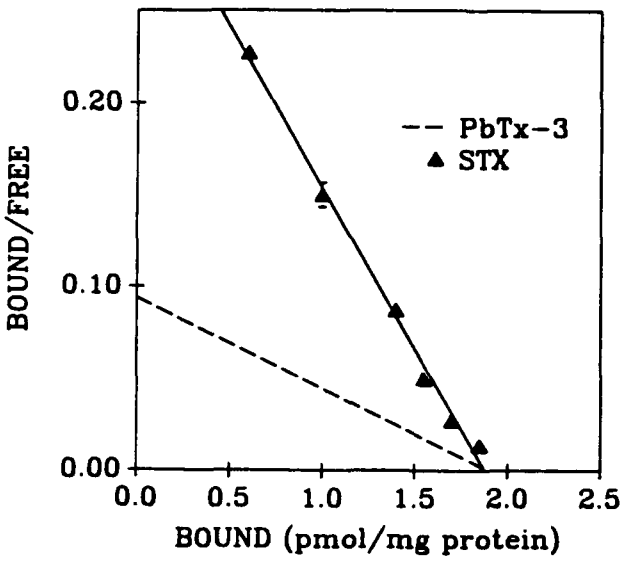

Figure 9. Scatchard analysis of high affinity binding for tritiated saxitoxin and tritiated brevetoxin. Experiments were performed using radiolabeled toxin at ranges of 0.78-25 nM. Data is representative of five experiments, each point being the mean of triplicates, error bars defining the range. Dashed and solid lines represent the LIGAND-derived high affinity binding for each toxin. Note equivalent B_{max} value in both cases indicating a 1:1 binding stoichiometry.

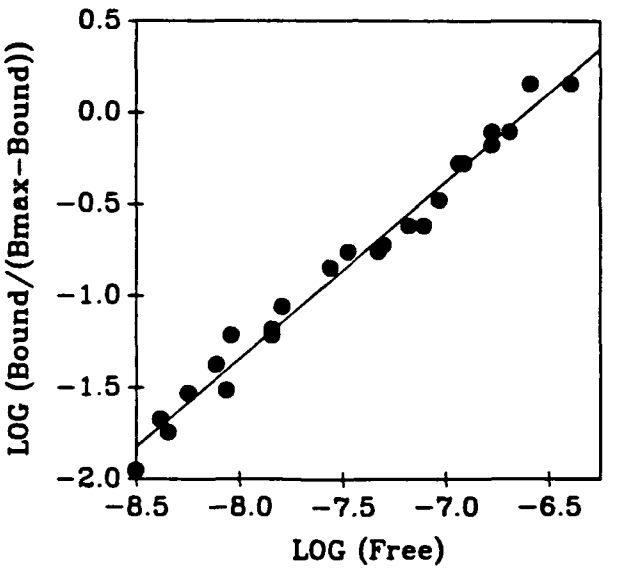

Figure 10. Hill plot of tritiated brevetoxin binding. Data from saturation isotherms were plotted and coefficients calculated. Each point represents the mean of triplicate determinations. The data points were pooled from three independent experiments, yielding a Hill coefficient of 1.00 $\pm$ 0.02.

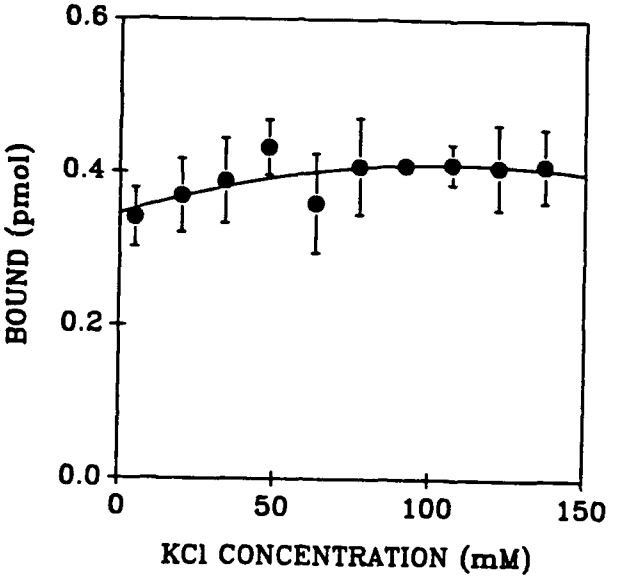

Figure 11. Voltage dependence of binding of tritiated brevetoxin. Specific binding of 10 nM radioactive toxin to synaptosomes was measured at the indicated concentrations of K+, as described under Experimental. KCL was exchanged for an equal concentration of choline chloride in stnadard binding medium. The membrane potential of synaptosomes was calculated to range from -55 mV in standard t 'nding medium, to 0 mV at 135 mM KCl. Data are representative of four replicates, the means of triplicates for each replicate. Error bars span 95% confidence limits.

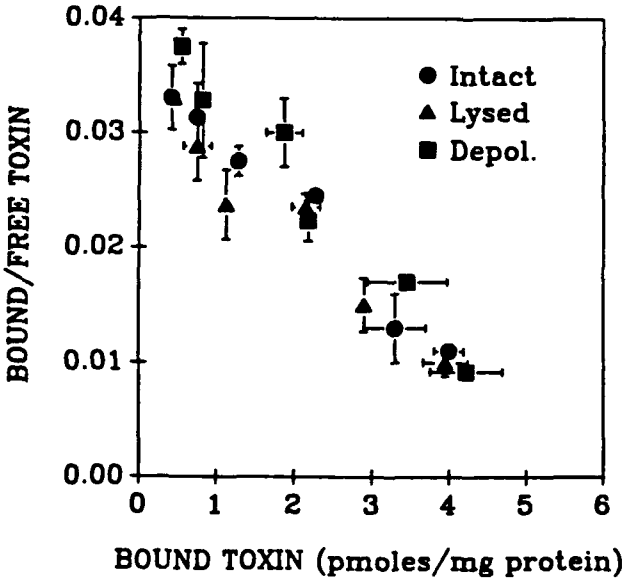

Figure 12. Scatchard analysis of specific brevetoxin binding to intact, lysed, and depolarized synaptosomes. Non-specific binding, measured in the presence of 10 μM unlabeled brevetoxin, was subtracted from the results. Results are result of four separate experiments, triplicates at each concentration value for each replicate.

Photoaffinity probe stability - Analysis of the p-azidobenzoyl photoaffinity probe by reverse phase HPLC (Microsorb, 5 μM, C18, 25 cm bed) was used to determine its purity and specific activity. Underivatized PbTx-3 showed a retention time of 8 min in 85% isocratic methanol (1.4 ml/min), whereas the p-azidobenzoyl brevetoxin demonstrated a retention time of 48.5 min. The unlinked p-azidobenzoic acid eluted with a retention time of 2 min (breakthrough volume). Carbodiimide coupling of p-azidobenzoic acid to [^{3}H]PbTx-3 was quantitative with respect to PbTx-3, resulting in a photoaffinity derivative with a specific activity of 10-13 Ci/mmol. The photoreactive derivative has been shown by HPLC and binding activity to be stable for at least 1 year when stored in 100% ethanol at -20°C.

Irradiation optimization - Through spectrophotometric analysis of UV irradiated p-azidobenzoic acid, it was determined that a 30 sec exposure to a wavelength of 254 nm was effective in completely activating the pure compound in a solution not containing synaptosomes. Photoaffinity probe binding to synaptosomes, with no prior BSA incubation or washes, was used to determine the optimal time needed for maximum total binding. Based upon both spectrophotometric analysis of activated p-azidobenzoic acid and information gained from the time course of photoaffinity probe binding to synaptosomes, five minutes was chosen as the irradiation time needed for optimization of specific binding activity.

Irradiation of synaptosomes at a 254 nm wavelength for 5 min, followed by incubation with 0.2 nM pAB [^{3}H]PbTx-3 for 1 hr at 4°C, centrifugation, and assay for tritium activity indicated that approximately 11% of binding capacity is lost upon UV exposure compared with unirradiated synaptosomes.

Binding studies using p-azidobenzoate brevetoxin - In the absence of irradiation, pAB [^{3}H]PbTx-3 was displaced in a competitive manner by PbTx-3 from its specific site of binding in synaptosomes, illustrating a common binding site for native and derivatized brevetoxin. Half-maximal equilibrium binding of the derivatized brevetoxin was observed at a concentration of 0.2 nM. Underivatized brevetoxin exhibits half-maximal binding at 1.9 nM ligand concentration.

The time necessary to dissociate one half the total unirradiated [^{3}H]PbTx3-Pho from its receptor ($t_{1/2}$) was determined by performing a binding experiment as described in Experimental Procedures. After this initial incubation, samples were centrifuged and resuspended in standard binding medium. The loss of total radioactivity at various time intervals was compared to a control incubation (time zero). The $t_{1/2}$ of the photoaffinity probe was approximately 30 min, a value comparable to the 20 min $t_{1/2}$ determined for native PbTx-3. These results indicate that there is sufficient time for the photoaffinity-labeled sodium channel to be washed in order to reduce nonspecific binding without substantial loss of specifically-bound photoaffinity probe.

A Rosenthal analysis of photoaffinity probe binding to synaptosomes resulted in a non-linear plot, suggesting the presence of two binding sites for the brevetoxin molecule in synaptosomes (Fig. 13). Binding of the photoaffinity label to the high affinity site resulted in a LIGAND-derived half maximal binding concentration (Kd) of 0.21 nM and maximal binding capacity (Bmax) of 2.12 pmol/mg protein. Low affinity site binding is characterized by a Kd of 50 nM and Bmax of 92 pmol/mg protein. The B_{max} for the high affinity site is similar to the concentration of sodium channels in synaptosomes while the B_{max} for the low affinity site greatly exceeds the concentration of sodium channels. Evidently, only the high affinity site represents binding to sodium channels. The inset to Figure 13 shows the LIGAND-derived saturation binding data for both high and low affinity brevetoxin binding sites. At the high affinity site Kd of 0.2 nM, greater than 75% of the total radioactivity is bound to the high affinity site. Since the high affinity low capacity site was the focus of this study, all receptor purification protocols were performed at concentrations of 0.2 nM pAB [^{3}H]PbTx-3.

Low Affinity Binding - Additional experiments were performed to affirm that the binding characteristics of the derivatized compound and the native ligand are similar. The lower Kd of the photoaffinity derivative (0.2 nM) compared with that of native brevetoxin (1.9 nM) may be due to the added hydrophobicity of the benzene ring, which is clearly evident in the substantially increased retention time on C18 reverse phase HPLC columns. Binding studies utilizing PbTx-3 to displace unirradiated [^{3}H]PbTx3-Pho from its membrane receptor demonstrated displacement patterns indicative of competitive inhibition. These results show that PbTx-3 and its p-azidobenzoyl derivative bind to the same receptor site on sodium channels.

Rosenthal analysis of the p-azidobenzoyl brevetoxin binding demonstrated the presence of two distinct binding sites. Previous work documented a single brevetoxin binding site in experiments which utilized radioligand concentrations up to 20 nM (Poli et al., 1986). Evidence for 2 site binding has been seen in

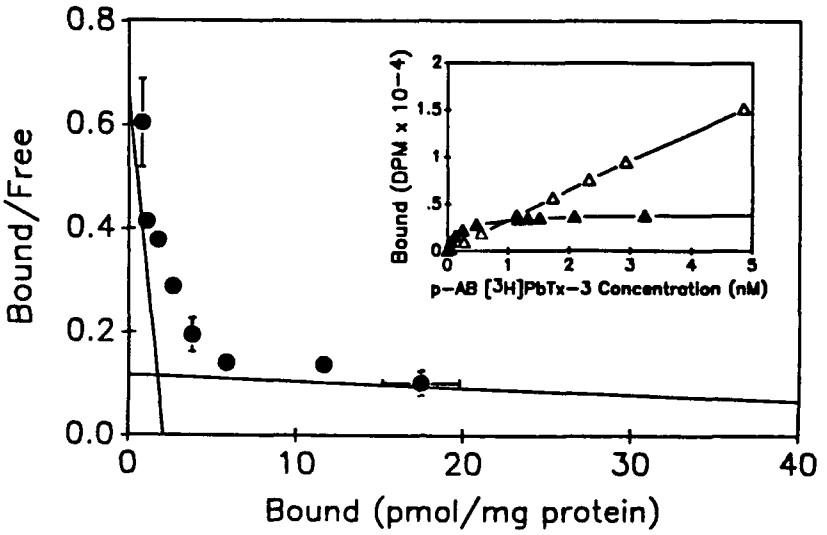

Figure 13. Rosenthal analysis of tritiated brevetoxin photoaffinity probe to crude synaptosomal preparations. Concentrations of photoaffinity probe ranged from 0-100 nM. Inset: LIGAND-derived saturation binding data for both the high affinity/low capacity and low capacity/high affinity brevetoxin binding sites. Binding experiments were carried out as described in Experimental. High affinity binding saturates at about 0.4 nM and low affinity binding saturates at approximately 150 nM (off-scale). Error bars span the range of duplicate values and points represent the mean (when not visible, error bars are smaller than the symbol size).

Rosenthal analyses of native brevetoxin which used radiolabeled toxin concentrations up to 175 nM (Edwards et al., 1991) as well as in competition binding studies with underivatized brevetoxins (Trainer et al., 1991).

In both types of experiment, low affinity binding became evident above 25 nM, a concentration of brevetoxin well beyond that at which the half maximal biological effect is observed (1.6 nM). In a study in which the same synaptosomal preparation was incubated with either [^{3}H]saxitoxin and [^{3}H]PbTx-3, the Bmax of saxitoxin and high affinity brevetoxin were found to be identical ($p < 0.01$). This indicates that saxitoxin and high affinity site brevetoxin binding occur in equivalent ratios and suggests that low concentrations of brevetoxin bind in a 1:1 stoichiometry with the sodium channel. In view of these results indicating a high affinity interaction of brevetoxin with the sodium channel, the high affinity low capacity site was the focus of the present study. Further work is in progress to confirm that the low affinity brevetoxin binding occurs on a separate protein component of synaptosomal preparations, as expected from its high B_{max} value.

Gel filtration - Gel filtration studies were performed under reducing and non-reducing conditions. Under non-reducing conditions, a single peak of protein and specifically-bound radioactivity (Fig. 14a) which corresponded to a Stokes radius of 55 $\pm$ 3 Å (n = 5) was eluted from the Sephacryl S-300 column. Cleavage of the α subunit from the disulfide-linked ß2 with 2-mercaptoethanol resulted in retention of binding at the high molecular weight protein band and gave better resolution of the solubilized proteins (Fig.14b)(Hartshorne et al., 1982). These results strongly suggest that pAB [^{3}H]PbTx-3 binds to the α subunit of the α-ß2 Na^+ channel subunit complex. Further treatment of column fractions showed that cleavage of terminal sialic acids using neuraminidase (not shown) resulted in no change in elution characteristics of the brevetoxin-binding protein, indicating that the brevetoxin is not covalently attached to sialic acid residues.

SDS-PAGE - SDS-PAGE analysis of pAB [^{3}H]PbTx-3 covalently bound to synaptosomes was performed using a 5-20% gradient gel as well as 5% gels. The gradient gels were used to resolve potential binding of the photoaffinity probe to both the α subunit (260 kDa), the ß2 subunit (33 kDa), and the ß1 subunit (36 kDa). Specific binding was determined as the difference between total and nonspecific components which were electrophoresed in parallel lanes under reducing conditions. A protein band with a molecular mass slightly

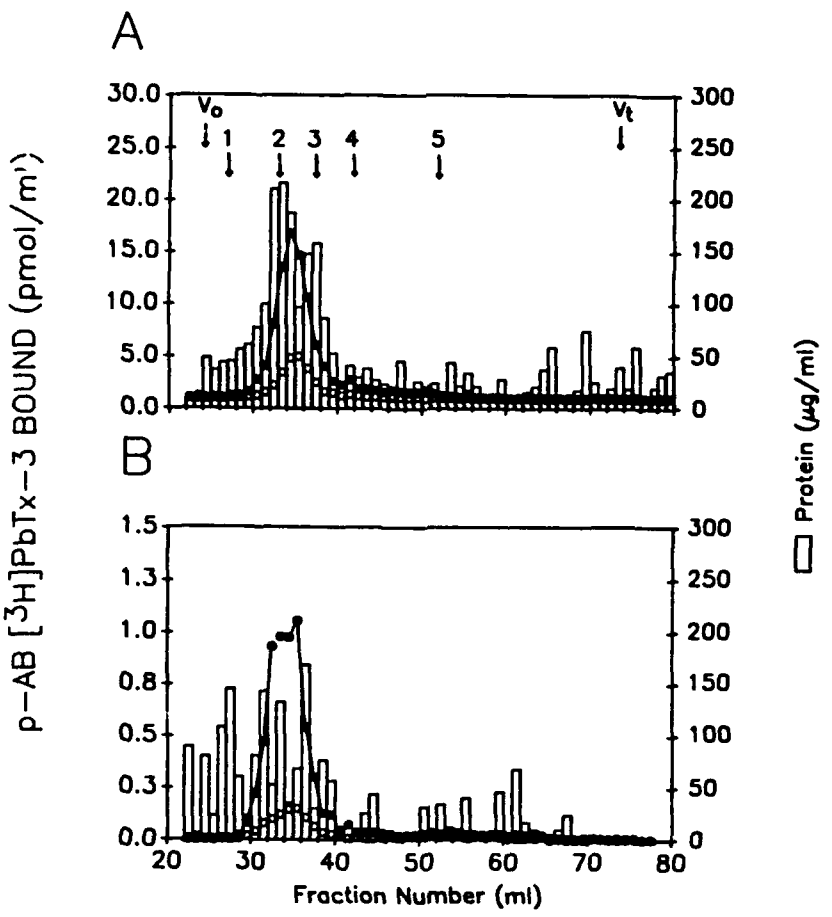

Figure 14. Gel filtration of the brevetoxin receptor. Measurements of total binding (closed circles), non-specific binding (open circles), and protein (bars) were made. Fraction volume was 1 mL. Molecular weight standards were: 1=thyroglobulin (669 kDa), 2=ferritin (440 kDa), 3=catalase (232 kDa), 4=aldolase (158 kDa), and 5=ovalbumin (43 kDa). Gel filtration chromatography was performed in both the absence (A) and presence (B) of 15 mM mercaptoethanol.

greater than the 200 kDa standard marker protein at about 260 kDa was determined to contain the most specifically-bound radioactivity (Fig.15a). Since this band was located in close proximity to the stacking gel which contained radiolabeled proteins unable to enter the resolving gel, a 5% gel was used to separate these two components and to provide further evidence of α subunit interaction with the radiolabeled photoaffinity probe (Fig 15b). SDS-PAGE indicated that approximately 20% of the specific binding component was covalently incorporated into a single 260 kDa protein following photolysis.

Immunoprecipitation - The antibody raised to the SP 1 region of the sodium channel α subunit was incubated with PbTx-Pho NaCh purified through affinity chromatography on wheat germ agglutinin-Sepharose. This antibody showed significant ($p < 0.05$), concentration-dependent recognition of the brevetoxin-linked Na^+ channel protein covalent conjugate over the preimmune serum control (Fig.16). These results further confirm that the 260 kDa protein labeled by pAB [^{3}H]PbTx-3 is the α subunit of the sodium channel.

Brevetoxin Binding Site Characterization Discussion

Brevetoxin Binding Experiments. Previous saturation binding experiments employing radiolabeled toxin concentrations from 0.78 nM to 25 nM yielded a Kd of 2.9 nM and a Bmax of 6.8 pmoles/mg protein. Scatchard analysis of these data resulted in regression lines which were slightly nonlinear. Further experiments in which the maximum concentration of radiolabeled toxin was increased to 175 nM yielded Scatchard regression lines that were noticeably nonlinear, suggesting multiple binding site classes.

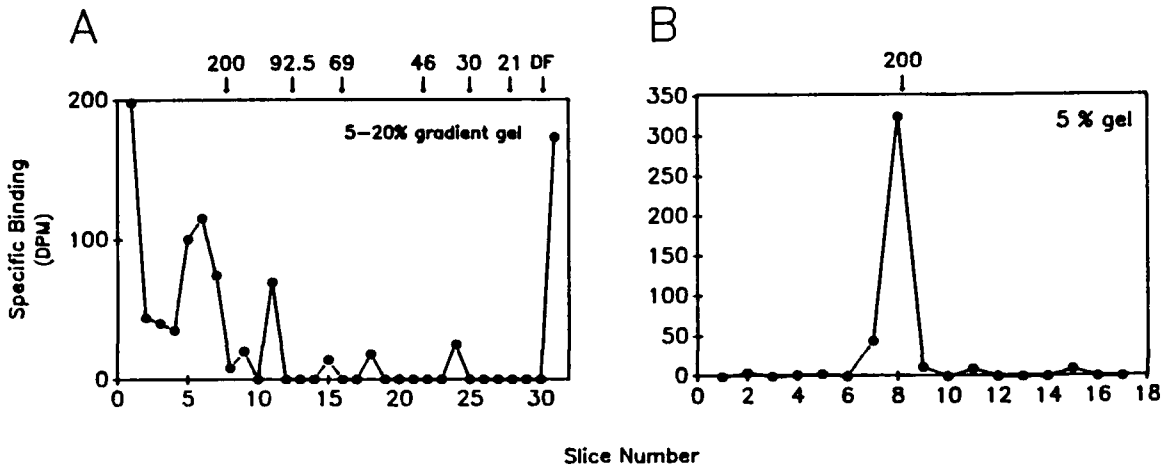

Figure 15. SDS-PAGE analysis of the covalently labeled brevetoxin receptor. Specific binding was calculated as described in Experimental, under reducing conditions. Molecular weights, designated in KdA on the upper abscissa, were determined by comparison with molecular weight markers in adjacent lanes. Gel slices were 4 mm thick. Slices were dissolved in Soluene and 0.1 mL hydrogen peroxide for three hours. Samples were counted in OCS counting fluid, comparing analyses on 5-20% gradient gels (A) or 5% gels (B). Stacking gel and dye front were not assayed for activity.

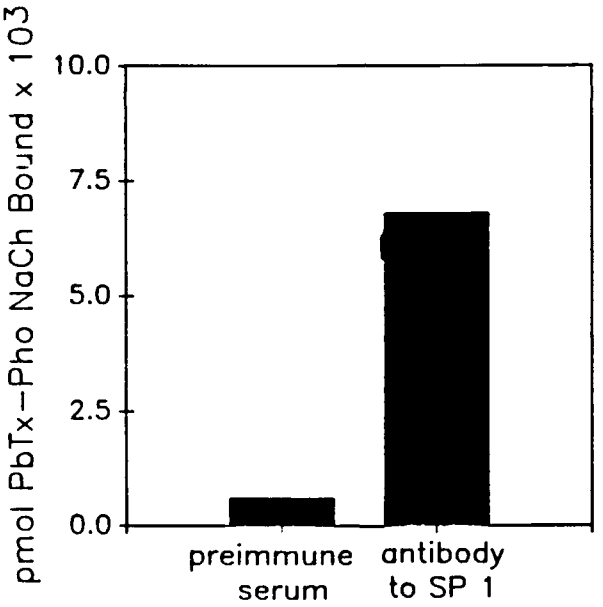

Figure 16. Immunoprecipitation of the covalently labeled brevetoxin receptor. Tritiated brevetoxin photoaffinity probe-sodium channel complex was solubilized from synaptosomes, purified through ion exchange and wheat germ agglutanin chromatography. Either pre-immune serum (left) or antibody to the SP1 portion of the α-subunit of sodium channel was used to attempt recognition. Protein concentrations were 0.3 mg preimmune serum or 0.1 mg purified antibody; 40 pmoles of sodium channel per tube. Samples were precipitated with protein A Sepharose and precipitates were counted for 20 minutes in scintillants.

LIGAND analysis of saturation binding data demonstrates a significantly better fit ($p < 0.001$) for a two binding site class model than for a one site model. Thus, saturation binding data analyzed by LIGAND indicates the presence of multiple classes of brevetoxin receptors possessing different binding characteristics. We believe that the previously calculated Kd and Bmax values are overestimates of the LIGAND-derived values for the HA/LC binding site class, since previous values reflect some combination of binding to both HA/LC and LA/HC site classes.

Brevetoxin Photoaffinity Probe Preincubation. Experiments were performed in which 5 nM unlabeled brevetoxin photoaffinity probe was bound to synaptosomes and linked covalently by UV irradiation. Saturation binding experiments were then performed using the pretreated synaptosomes followed by Scatchard and LIGAND analyses.

Data from control experiments demonstrating photoaffinity probe binding to synaptosomes were similar to those in Figure 2 since a better fit for a two-site model was observed. The Bmax values for the two experiments are not statistically different ($p < 0.01$). In contrast, photoaffinity probe binding exhibits much higher binding affinity (lower Kd) than the native toxin. This is likely due to the greater hydrophobicity of the brevetoxin photoaffinity probe, clearly illustrated by its greater retention time relative to native brevetoxin on reverse phase HPLC.

Pretreatment of synaptosomes with unlabeled photoaffinity probe, results in a linear Scatchard plot due to the blockage of HA/LC site binding by the covalently-linked photoaffinity derivative. This experiment also supports evidence for multiple binding site classes for the following reasons. If binding sites with uniform Kd values were present, modification of a portion of the total number of sites using the photoaffinity probe would result in a Scatchard regression line parallel to that of control experiments, but with a decreased Bmax. Conversely, if two or more classes of sites possessing dissimilar Kd values were present, the 5 nM unlabeled photoaffinity probe would saturate the HA/LC sites (Kd = 0.2 nM) resulting in their blockage following UV irradiation. Our data support the latter model since Scatchard analysis demonstrates a loss in HA/LC binding. We conclude that radiolabeled toxin binding to the HA/LC site was blocked by pretreatment with brevetoxin photoaffinity probe, and that only binding to the LA/HC site class was observed.

Brevetoxin:Saxitoxin Binding Ratio. Saxitoxin binds to site 1 associated with the VSSC at a ratio of one toxin molecule per channel (Hille, 1984), and binds to all VSSC present in normal adult mammalian brain tissue (Haimovich et al., 1986; Sherman et al., 1983; Yarowsky et al., 1989). Previously, it was assumed that brevetoxin bound to site 5 in a similar manner. Historical comparison of the saxitoxin binding maximum (approximately 1.4 - 4.4 pmol/mg protein, Catteral et al., 1981) with that derived for brevetoxin (6.9 pmol/mg protein, Poli et al., 1986) indicated that the binding sites for these neurotoxins were not present in equal numbers. This suggested that either saxitoxin does not bind to all of the VSSC present, or that brevetoxin binds to additional sites which do not also bind saxitoxin. In parallel binding assays, it was determined that the brevetoxin HA/LC binding site class Bmax derived from LIGAND (1.94 $\pm$ 0.98 pmol/mg protein) was not statistically different from the saxitoxin Bmax value (1.72 $\pm$ 0.72 pmol/mg protein), indicating that saxitoxin binding and high affinity brevetoxin binding (HA/LC) are stoichiometrically equivalent. Batrachotoxin (Catterall, 1985; Catterall et al., 1981), saxitoxin and brevetoxin HA/LC site class binding all exhibit Bmax values of approximately 2 pmoles/mg protein, suggesting thier association with the VSSC in a 1:1:1 ratio.

Low Affinity Binding Site Characterization. In presenting evidence of multiple binding site classes, it is necessary to investigate other possible reasons for the apparent changes in brevetoxin binding affinity. Experimental artifacts due to the physical properties of the toxins, as well as changes in membrane binding capabilities brought about by specific toxin binding, must both be considered as possible causes of the LA/HC binding at high toxinconcentrations.

Solvent effects, changes in membrane fluidity and brevetoxin adsorption are all possible complicating factors. The influence of solvent effects and nonspecific brevetoxin adsorption, to either plastic vials or membrane components, is effectively eliminated by defining specific binding as the difference between total binding and nonspecific binding values. Toxin partitioning into the membrane lipid milieu could result in membrane lipid fluidity changes and altered toxin binding affinity. This would be manifest as a change in the Kd value with no alteration in the Bmax value (membrane-associated toxin would be subtracted as nonspecific). What is seen, however, is an increase in Kd associated with an increased Bmax. This argues against the

alteration of binding affinity as a result of a nonspecific association of the hydrophobic toxin molecule with the lipid bilayer.

Brevetoxin binding to the VSSC, causing subsequent activation, results in membrane depolarization due to transmembrane ion flux. It is possible that the change in membrane polarity alters the binding characteristics at high brevetoxin concentrations. Additionally, the binding of toxin molecules to receptor sites might bring about structural changes which either enhance or inhibit subsequent toxin binding to other sites. The possibility of brevetoxin-binding dependence on membrane depolarization and site allostery were examined.

Hill plots yielded regression lines with slopes of 1.00 $\pm$ 0.02 (n=4). This indicates the lack of cooperativity between the classes of brevetoxin binding sites. Thus, we conclude that the binding of brevetoxin molecules does not alter subsequent toxin binding through allosteric modulation, since this would be evidenced by a Hill coefficient significantly different from 1.

Brevetoxin saturation binding experiments performed in the presence of 1 μM tetrodotoxin (to inhibit membrane depolarization) gave similar results to those performed in the absence of tetrodotoxin. Further examination of brevetoxin binding equilibria in synaptosomes (n=3, for lysed, intact, and depolarized conditions) has established to our satisfaction that there is no statistically-significant difference in Bmax or Kd between control and altered conditions, nor is there any marked variability in specific binding with increasing KCl depolarization at a fixed [^{3}H]PbTx-3 concentration). These observations demonstrate that the binding of brevetoxin PbTx-3 is not membrane-potential dependent.

Thus, the LA/HC site class does not appear be a binding artifact due to the physical properties of the brevetoxins. There is no allosteric interaction between brevetoxin binding sites or binding site classes. The LA/HC site class is not simply a modification of the HA/LC site class induced by increasing concentrations of the depolarizing brevetoxins. We conclude, therefore, that LA/HC brevetoxin binding occurs at a separate specific binding site class.

Binding Site Class Function. The depolarization of nerve membrane by brevetoxin (PbTx-3) has been shown to be half-maximal at 1.6 nM (Huang et al., 1984) and the LD50 value of brevetoxin toxicity in fish bioassay is approximately 4 nM (Baden et al., 1988). Thus, brevetoxin concentrations in the low nanomolar range are sufficient to elicit both physiological and toxic effects. This suggests that the HA/LC brevetoxin-binding component associated with the VSSC is responsible for physiological activity. Recent work has localized the brevetoxin HA/LC site class to domain IV on the sodium channel α-subunit (Trainer et al., 1991). Further experiments, including peptide mapping by comparison to known α-subunit sequence, will aid in development of a model of HA/LC brevetoxin binding effects on sodium channel gating kinetics.

The physiological significance of brevetoxin binding to the LA/HC site has not yet been demonstrated. Binding affinity values in the nanomolar range, the relatively small number of binding sites and the displacement of radioligand by unlabeled toxin suggest that a specific toxin-receptor interaction is occurring at this LA/HC site (Burt, 1985). Some insight into the effect of brevetoxin binding at the LA/HC site may be obtained from experiments which investigate allostery among sodium channel receptor sites. Allosteric modulation of neurotoxin binding to sites 2 and 4 by brevetoxin (Sharkey et al., 1987), has been shown to be half-maximal at PbTx-2 concentrations greater than or equal to 20 nM, a value substantially greater than the HA/LC Kd of approximately 2 nM. It is plausible that the observed allosteric effects of brevetoxin on neurotoxin binding to sites 2 and 4 may be due to their interaction with the LA/HC site class receptor. Further work is necessary to determine whether tha LA/HC binding site class is physiologically active, and whether the multiple binding site classes are located on different cell types, different ion channel types, different sodium channel subtypes (Gordon et al., 1987) or different sites on the same VSSC.

Binding Site Purification - A necessary component of successful receptor isolation is the ability to assay toxin binding activity at all stages of purification. Na^+ channels that have been solubilized from neuronal tissue with nonionic detergents retain neurotoxin binding activity at receptor site 1, but not at sites 2 and 3 (Catterall et al., 1979). Similarly, preliminary work indicated that specific binding could not be effectively monitored by incubation of tritiated brevetoxin with detergent-solubilized membrane preparations; the hydrophobic interactions of the toxin with its solubilized receptor appear to be largely nonspecific (Poli et al., 1986). Thus, it was necessary to develop a radiolabeled photoaffinity probe which could be covalently linked to the receptor site in synaptosomes prior to solubilization, allowing for assay of the receptor throughout the

purification protocol.

The size estimate of the tritiated, photoaffinity-labeled Na^+ channel eluted from a Sephacryl S-300 column (55 Å) is an indication that the photoaffinity label is incorporated into a large protein/detergent complex of similar size to the sodium channel (Hartshorne et al., 1982). Separation of the covalently-bound α and ß2 subunits with 2-mercaptoethanol resulted in no significant change in elution profile, indicating that the brevetoxin photoaffinity probe preferentially binds to the α subunit. Due to the low resolution capability of the gel, a shift in molecular weight was not observed upon treatment with either 2-mercaptoethanol or neuraminidase, therefore this method was used only to give a molecular weight approximation.

The Na^+ channel α subunit from rat brain contains more than 20% (w/w) carbohydrate (Messner and Catterall, 1985), approximately 12% of which is negatively-charged sialic acids. More than 100 sialic acid residues are believed to surround the Na^+ channel pore in an approximate spherical volume of 75 Å. It has been suggested that sialic acids may play an important role in channel activation through their electrostatic influence on membrane potential because removal of sialic acids alters the voltage-dependence and subconductance states of purified and reconstituted sodium channels. The potential reactivity of the p-azidobenzoyl brevetoxin with these sugar molecules was tested by specific cleavage of sialic acid residues with neuraminidase. Treatment of PbTx-Pho NaCh with this enzyme resulted in no change in elution pattern of the tritiated complex by gel filtration, demonstrating that terminal sialic acids do not bind to the photoreactive compound.

Immunoprecipitation of the covalently-linked brevetoxin-sodium channel complex with an anti-peptide antibody directed against a segment of the α subunit conclusively demonstrated covalent incorporation of this neurotoxin to the Na^+ channel α subunit. The location of the brevetoxin receptor site on the α subunit correlates well with previous findings which demonstrate the importance of this subunit in channel gating and voltage sensing mechanisms. It has also been shown to contain binding sites for other neurotoxins, such as tetrodotoxin, saxitoxin, and α- scorpion toxins which are known to specifically alter normal Na^+ channel function.

The tritiated p-azidobenzoyl brevetoxin photoaffinity derivative, which forms a covalent bond with its membrane receptor upon UV irradiation, will be useful for assay of the ligand-receptor complex through all stages of purification. Since the binding site of [^{3}H]PbTx3-Pho has been shown to be identical to the receptor with which native brevetoxin interacts, the mapping of this covalently-bound ligand to its receptor sequence may ultimately result in precise localization of Na^+ channel receptor site 5. Knowledge of the position of this binding site may increase our understanding of the molecular mechanism of channel activation, a process altered by interaction of brevetoxin with a specific portion of the Na^+ channel α subunit.

Brevetoxin Binding Site Modeling Results and Discussion

Much of the pharmacological data available on the brevetoxins surrounds PbTx-3, the allylic alcohol derivative of the PbTx-2 class. Some correlations in the literature (Huang et al., 1984, and references therein) show that within a structural class of brevetoxin, i.e. PbTx-2 type or PbTx-1 type, minor substituents play an insignificant role in affecting toxicity or inhibition constant binding character. However, there was shown to be a statistically significant difference between the two brevetoxin classes. By using radioactive derivatives of either PbTx-1 or PbTx-2, specific binding parameters such as binding affinity (expressed as K_D, the dissociation constant) and number of sites (B_{max}, binding maximum in pmol/mg synaptosomal protein), it is possible to compare directly the relative binding affinities and maxima. Moreover, derived values for ciguatoxin dissociation constants and binding maxima can be calculated, there being no radioactively labeled material available for direct binding studies.

Purified natural toxins within either the PbTx-2 or PbTx-1 toxin class used as competitive inhibitors of radioactive toxin of the same class failed to show any statistical correlation, i.e. all class PbTx-2 toxins exhibited inhibition constants equivalent to one another. PbTx-1 toxins efficiently displace tritiated PbTx-2 type toxins from site 5 of the VSSC, indicating that PbTx-1 brevetoxins bind with a higher affinity to the specific binding site than do PbTx-2 type toxins. Ciguatoxin, by our studies and by literature accounts, competitively inhibits brevetoxin binding at much lower concentrations than either of the brevetoxin classes. Inhibition constants for ciguatoxin displacing PbTx-2 type brevetoxin from its specific site are in the 0.1-0.2 nM range.

Thus, the dissociation constants calculated for each of the three toxin types progress from the least potent PbTx-2 class at 2.6 nM, to the PbTx-1 class at 0.8-1.0 nM, and culminate with ciguatoxin, displaying a calculated dissociation constant of 0.1 nM. Mouse intraperitoneal bioassay data progress in exactly the same

manner, although the magnitude of the derived values exceeds the differences in specific binding data. As expected, the inhibition constant data parallels the dissociation constant and mouse bioassay data. Retention times on C-18 reverse phase HPLC columns indicate that hydrophobicity increases from PbTx-2 type to PbTx-1 type to ciguatoxin.

That all of this data correlates seems to indicate that toxicity is related to binding affinity, which in turn correlates with relative hydrophobicity. That the binding maximum does not vary from toxin type to toxin type is reassuring, for it indicates that specific site 5 behaves like a homogeneous binding domain when exposed to these toxins, and further, that we cannot distinguish sub-populations of high affinity binding sites. It is the pharmacological and bioassay data that we seek to correlate with the structural characteristics of each toxin type as determined by computer-aided molecular mechanics.

The topography involved in binding and expression of activity by the polyether ladder toxins can be modeled as activity (A, triangles) and binding (B, squares) loci on the toxins, as shown in figure 17. The inherent flexibility of each toxin permits either more (c) or less (b, then a) effective simultaneous interaction of the A and B domains with specific sites on the VSSC. A cursory examination of the brevetoxin and ciguatoxin structures in figure 17 reveals that PbTx-2 has 16 potentially rotatable bonds, PbTx-1 has 31, and ciguatoxin has over 40.

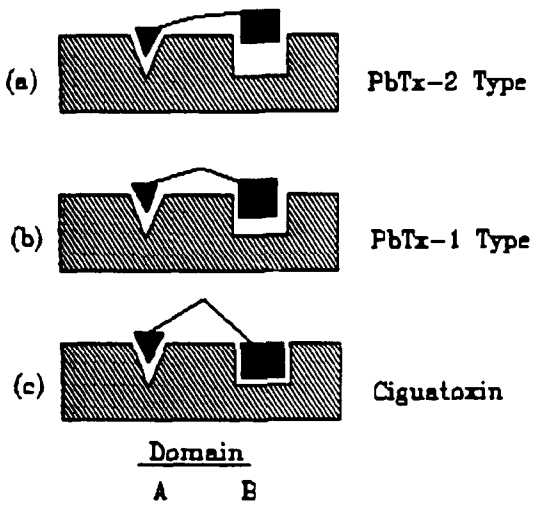

Figure 17. Schematic model of polyether toxin interaction with VSSC. Toxin flexibility increases from (a) to (b) to (c) and correlates with increasing potency. It is proposed that increased flexibiity allows for morre favorable interaction of binding (B) with activity (A) centers located on distal ends of each toxin.

While our long-term goal is to evaluate the conformational space of all three polycther ladder toxin types, we have begun our efforts with the most rigid of the three types. Examination of the structures suggested that PbTx-2 would be most rigid. Indeed, Lin et al. (1981) originally described the structure as a "rigid ladder-like structure".

Internal coordinate Monte Carlo modeling of the G-H-I rings, in which all torsions of the 8-membered ring are varied except for the double bond in the two ring fusion bonds, reveals only two low energy conformers. These differ by 4.06 kcal/mol which corresponds to greater than 99.9% of the lower energy one.

By contrast, modeling of the C-D-E-F rings shows a number of conformers and suggests that the toxin is not rigid at all. Stereo views of the seven lowest energy isomers and their Boltzmann populations are shown in figure 5. Note that the four lowest energy structures, which differ by only 1.6 kcal/mol, comprise greater than 99% of the total population. Note also that structures 2 and 4 possess an approximate 90Å bend at the D-ring.

With these preliminary results in hand, we used the internal coordinate Monte Carlo technique to probe the conformations of PbTx-2. Although the complete details of these calculations will be reported elsewhere,

the conformations of PbTx-2. Although the complete details of these calculations will be reported elsewhere,

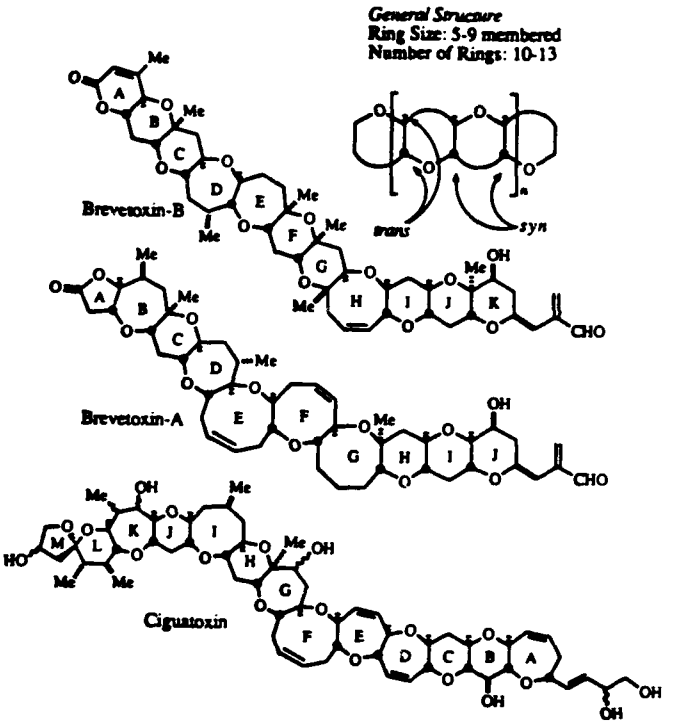

Figure 18. The polyether marine toxin PbTx-2, PbTx-1, and ciguatoxin, together with a schematic general structure of the "ladder" configuration which indicates their structural similarity.

the method did locate a number of conformations, including all four of the low energy C-D-E-F conformations corresponding to structures 1-4 of figure 19. Interestingly, the relative positions of the three lowest energy conformations were scrambled due to transannular interactions not present in the models. For example, the lowest energy conformation of the toxin contains the D-E configuration corresponding to conformation 3 in figure 19. Conformation 1 of figure 19 appears next, at 0.26 kcal/mol above the global minimum. Then comes conformer 2, at 0.57 kcal/mol above global minimum and conformer 4 at 1.82 kcal/mol above the global minimum. Figure 20 illustrates the superimposition of the global minimum and the 0.57 kcal/mol structures for PbTx-2 and preliminary results for ciguatoxin, and illustrates the dramatic effect of specific ring flexibility on the position of the A-ring relative to the stationary distal ring in each case. The A-ring sweeps over 12 angstroms by this single ring flip of the C-D rings in PbTx-2, and about 35 Å in ciguatoxin.

The 4 brevetoxin structures comprise greater than 98% of the total population and we would expect that within this composite population lies a conformation or conformations that are "active" with respect to VSSC interaction.

Monte Carlo modeling of PbTx-1 is proving problematic, but preliminary manipulation has yielded two low energy conformers which exhibit a 25Å travel in the A-ring.

As neurotoxins, the polyether ladder toxins interact with the VSSC based primarily on their binding to a single specific site called site 5, located on the a-subunit of the channel. Unpublished photoaffinity probe results indicate that brevetoxin binds in a 1:1 stoichiometry to Domain IV of the channel (Trainer et al., 1991).

We propose that brevetoxins interact with the a-subunit of the voltage-sensitive sodium channel by inhibiting free movement of the sliding helix (S4 region) as follows: (1) the toxin flexes to wrap around, against, or into its binding site, and is held in place by a combination of hydrophobic interactions, and perhaps hydrogen bondings; (2) conformational restrictions or perhaps reaction of an exposed lysine on S4 with an electrophilic site on the end of the toxin (such as the lactone or enal of the brevetoxins) might immobilize the sliding helix and favor channel opening.

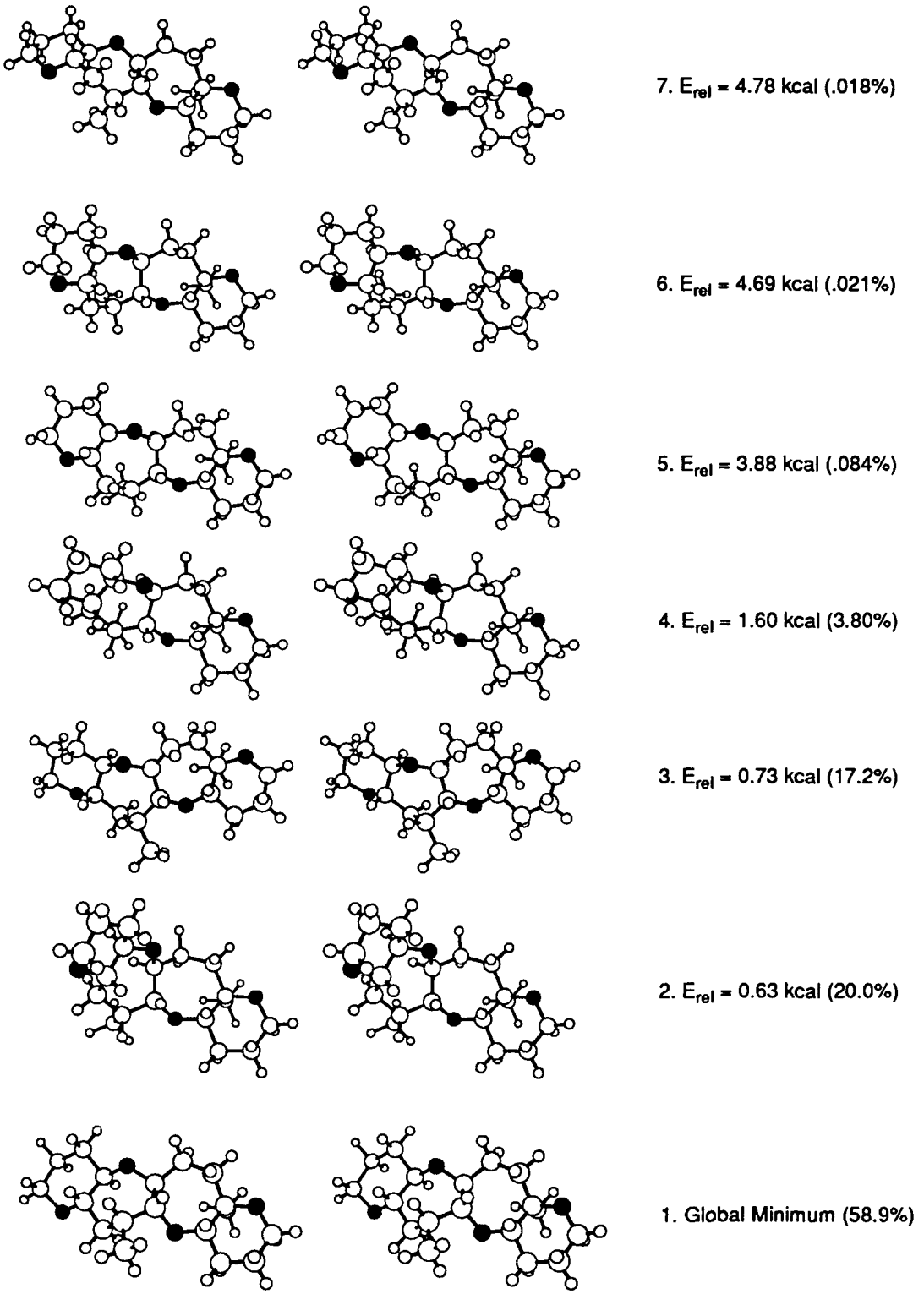

Figure 19. Conformational analysis of the C-D-E-F ring region of PbTx-2. Stereoviews of the 7 lowest energy conformers of the model compounds, with relative energies and Boltzmann distribution of each at room temperature (gas phase).

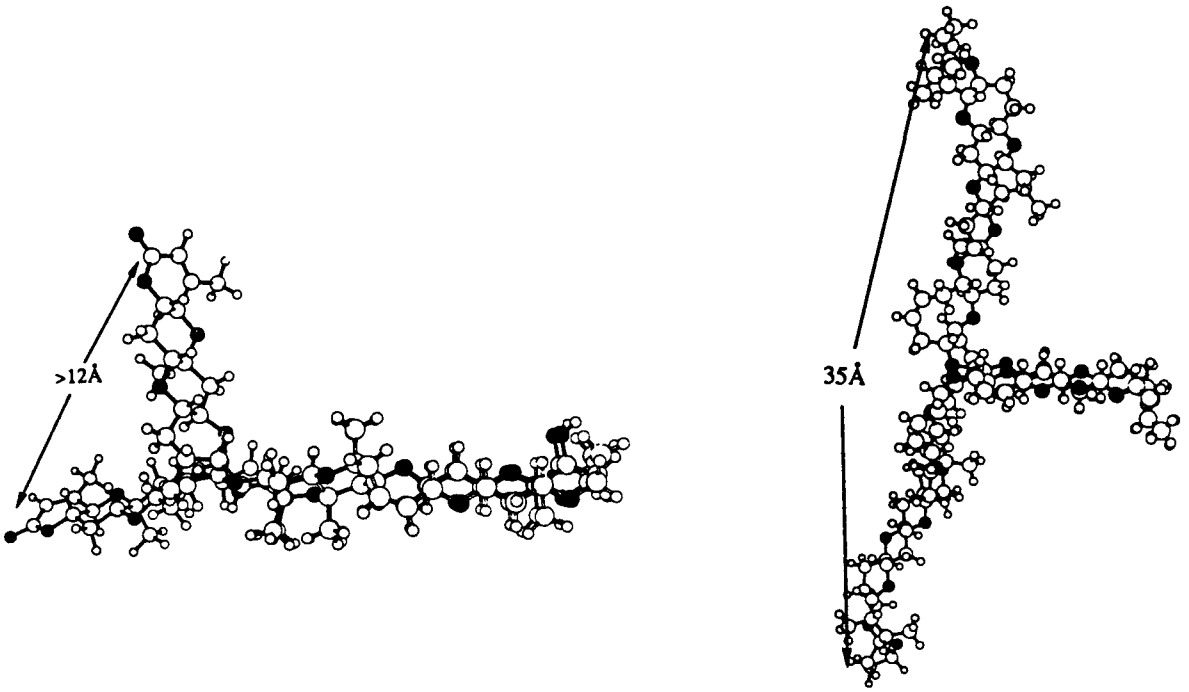

Figure 20. Superimposed conformers of polyether toxins. (Left) The two PbTx-2 structures correspond to the global instant minimum (extended) and the +0.57 kcal.mmole (bent) structure. The K ring is on the right side. (Right) By flexing the E-F-G region of ciguatoxin, and differing in energy by 0.8 kcal/mole, a 35 angstrom travel sweep is realized for the M ring. The mostly rigid A-E rings are located on the right side.

These separate events may correspond to the activity and binding functions illustrated in Figure 4. We believe both steps are required for the full expression of polyether ladder toxin action. This model is consistent with several intriguing facts: Squid or crayfish axons exposed to toxin exhibit depolarization which can be reversed by washing, but the reversibility is dependent on the length of exposure; upon binding to voltage clamped neurons, closed channels were opened and channel kinetics were modulated to exhibit slow inactivation kinetics (11); toxins administered to VSSC in rat brain synaptosomes can be reversibly bound if washout occurs within 20 min to 1 hr of application (8); and all three toxin types exhibit competitive displacement equilibrium binding constants in radioactive brevetoxin Rosenthal Analyses (8). Interestingly, the reversibility of toxin action is lessened for the more flexible toxins.

Our hypothesis is that the increased flexibility is reflected in the binding step described above. Note also that the calculated differences in energy for the toxin conformers allow significant populations of the bent isomers even in the absence of external influences. Moreover, differences of 2-3 kcal/mol could be easily overcome by hydrophobic interactions or hydrogen bonding, which might significantly stabilize a higher energy conformer relative to the calculated global minimum.

In summary, this working model describes the computational, pharmacological and binding results thus far generated, and consolidates our thoughts on the specific interaction between these toxins and the VSSC. Systematic chemical modification of the natural toxins is in progress as is the synthesis of toxin surrogates utilizing the modeling results as guideposts. We anticipate fully developing quantitative structure-activity relationships (QSAR) for polyether ladder toxins which will aid in the complete description of voltage-sensitive sodium channel topography and toxinology.

Toxin Derivatives and Deliverables

Affinity Columns. The mid-term report for DAMD17-88-8148 contained detailed protocols for the manufacture of brevetoxin PbTx-3 aminohexyl Sepharose columns (Trainer and Baden, 1991), based on the condensation of PbTx-3 succinate with H_2N-hexyl Sepharose in the presence of EDC or CDC. This condensation is the identical synthetic scheme we have used to produce protein-linked toxin for antibody production (Baden et al., 1984; Trainer and Baden, 1991). Yield for this protocol was 3-4 μmoles of toxin bound per mL of gel. Utilizing carboxyhexyl Sepharose, and appropriate carbonyldiimidazole coupling procedures, a general coupling procedure for toxins possessing an alcohol function has been developed.

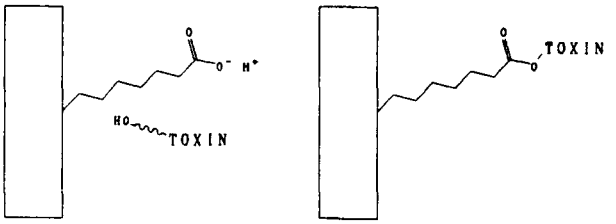

Figure 21. Synthesis schematic for coupling toxins containing alcohol functions to carboxyhexyl Sepharose.

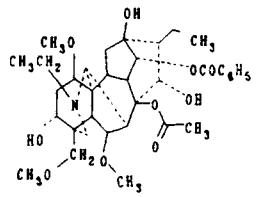

Figure 22. Aconitine (site 2 agonist).

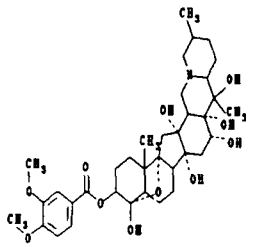

Figure 23.Veratridine (Site 2 agonist).

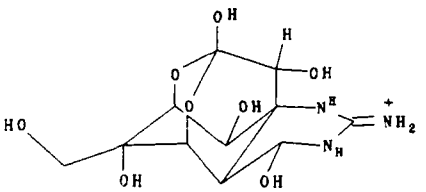

Figure 24. Tetrodotoxin (Site 1 blocker).

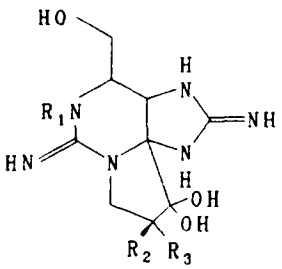

Figure 25. Decarbamoyl saxitoxin (Site 1 blocker)

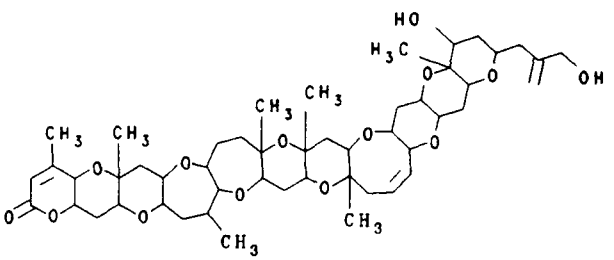

Figure 26. Brevetoxn PbTx-3 (Site 5 agonist).

Affinity column matrices are packed in organic preservatives like propylene glycol, and it is necessary to remove these items prior to utilization as an affinity matrix. Preservatives can be efficiently removed by washing on a scintered glass filter, first with 0.5 N NaCl solution fololowed by distilled water, methanol, and acetone. The final wash with acetone is as much to dehydrate the gel as it is to clean remaining preservative. Little difference in efficiency of coupling was discovered at this stage.

Gel matrices can be lyophilized at this point to completely dehydrate them, but it has been found that sufficient water is removed by the acetone rinse, and cleaned matrices can be dried with a strema of warm air prior to coupling. Dried CH- Sepharose resuspended in minimal dry benzene should be stirred or swirled for a few minutes prior to addition of carbonyldiimidazole. It is imperative that excess carbonyldiimidazole be added to fully activate all carboxy functions prior to addition of toxin. Excess CDI does not interfere with later reaction steps.

In all cases, affinity matrix was present in at least 6 X molar excess, to favor toxin coupling. Depending on the toxin (and hence the reactivity of the hydroxyl function) reaction temperatures varied. As a general rule, 70°C was sufficient to allow reaction of the most stubborn of hydroxyls. As a technical note, it is important that reaction mixtures be maintained in suspension (Sepharose is a solid) by swirling, not stir bar. In the latter case, complete pulverization of the solid matrix occurs. Following reaction, the remainder of the activated carboxy groups can be blocked with methyl or ethyl functions (by adding methanol or ethanol) or can be returned to their carboxy form (by water washing). Methyl blocked functional groups appears to be best for retaining a slightly hydrophobic nature to the column, and this should provide less influence on separations that does free carboxyl functions.

Each of the toxins, save tetrodotoxin, depicted on page 29 were succesfully linked to carboxyhexyl sepharose in virtual stoichiometric amounts. Deliverables of these affinity matrices has been made. Aconitine is provided as an additional non-contracted derivative. Tetrodotoxin has proved more difficult, principally because there are a large number of reactive hydroxyls in the molecule. Hence, protection reaction sequences were necessary. These synthetic measures were described in our mid-term report (Baden, 1990), and we have not progressed substantially in this area due to lack of toxin (it is purchased, not obtained from cultures).

Photoaffinity probes. Using an identical procedure, but substituting the carboxy function of p-azidobenzoci acid for carboxyhexyl sepharose, we provided complete unlabeled photoaffinity probes of each of the toxin except tetrodotoxin. These also have been supplied as Deliverables in the amounts specified. Again, aconitine is provided in addition. As soon as labeled toxins are available, the corresponding photoaffinity probes will be synthesized and delivered. The delay is due to a pre-requisite for tritium labeled methyl iodide, which is on back order and is due in mid-September. Tritiated brevetoxin photoaffinity probe has been delivered. Photoaffinity probes, as described in the mid-term report (Baden 1990), have not been successful as originally proposed. Two separate attempts to stabilize preparations failed. These attempts are described below under Sginificant Negative Results. Also proposed under that section, is an alternative to produce labeled material for completion of anticipated Deliverables.

Radioactive Toxins. ^{14}C-labeled toxins. As described in Experimental, brevetoxin, saxitoxin, and okadaic acid and their derivatives are available in radioactive carbon-form. Specific activity calculations have not been made with the latter two toxins (not contracted as Deliverables), but in the case of brevetoxin PbTx-3 the specific acitivity is 175 mCi/mmole $\pm$ 10 mCi/mmole. The specific activity is 17-fold higher than the contracted minimum specific activity. Shipment shall occur during the week of 16 September. The other toxins are available as requested.

Tritium-Labeled Toxins. The production of these derivatives is essential for the complete synthesis of labeled photoaffinity probes. We have made excellent progress in the area of brevetoxin derivatization to produce labeled probes, and these were described in detail in the mid-term report. Also described therein were the steps in derivatization necessary to produce labeled forms of tetrodotoxin and saxitoxin, and we feel we have made substantial progress with these extremely difficult organic molecules.

Tetrodotoxin and saxitoxin were approached from similar synthetic schemes, in each case utilizing the

primary hydroxyl either available (TTX) or approachable (STX through decarbamylation). In both cases our efforts were hampered by limited quantities of starting materials. Tetrodotoxin is available commercially and was purchased in 50 mg lots. As our work progressed we discovered that the less expensive citrated TTX was unsuitable for anything but the most crude electrophysiology work. Its purity was approximately 50% by our criteria. A more expensive product (and hence even more scarce) proved useful and we succeeded in obtaining reasonable yields of compounds (B), (C), and (D) of figure 27 for further work.

There are particular quirks to working with the TTX molecule, not the least of which is the difficulty in visualizing it on thin-layer chormatography plates during work-up between steps.

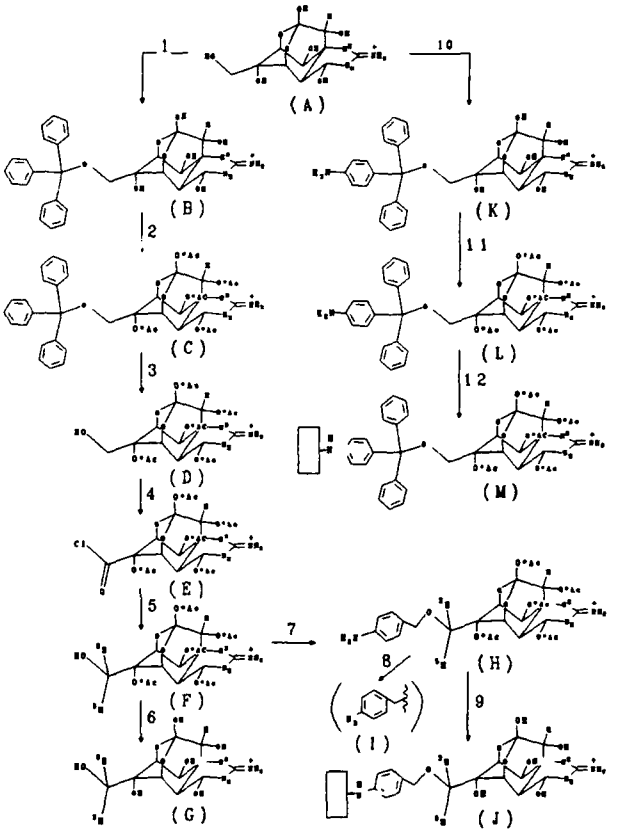

Figure 27. Derivatization scheme for tetrodotoxin. Tetrodotoxin (A) can be tritylated using trityl chloride in organic solvent (reaction 1) to produce primary hydroxyl protected tetrodotoxin (B). A proposed substitution reaction using aminotrityl chloride (reaction 10) can yield compound (K), aminotrityl-TTX. Compound (B) non-primary alcohol functions are protected using acetylation in reaction (2) to yield (C), which can then be deprotected in dilute acid to yield (D) via reaction (3). From Compound (E), the acid chloride, to tritiated acetylated ttx (F) to its catalytic hydrogentation product (G) [reactions 4,5,6] and reactions 7 and 8, these are proposed but not complete and require further synthetic investigation. Likewise, reactions 11 and 12 are incomplete.

We circumvented this problem in two ways. First, the initial step in all our derivatization schemes made use of the trityl (triphenyl) chloride protection of the primary alcohol function (B) or (K). The aromatic nature of the protecting group provides a substantial uv-absorbing chromaphore ideal for seeing on fluorescent tlc plates. It also tends to become a derivative exquisitely soluble in organic solvents, ideal for further manipulation.

A second technique which has been utlized is shown in step (2) and (11), the protection of the secondary hydroxyls with ^{14}C acetate. This provides an excellent low level counting system for steps after cleavage of the

uv-active chromaphore and is deal for final assessment of cleavage of the acetate protecting groups at the end of the synthetic scheme. Hence, compounds (B),(C) and (K),(L) are uv-absorbing, (C), (L) and (M) are also radioactive in addition to their ultraviolet absorbance, compounds (D) and (E) are ^{14}C-labeled solely, compounds (F) and (H) are dual labeled with ^{14}C and ^{3}H, and compounds (G) and (J) are solely tritium-labeled. Deprotected primary TTX (D) is the stage in intermediate preparation for which we feel we have optimized work. Approximately two years of work was required to select and test the appropriate protecting and deprotecting groups, none of which is noteworthy except for the sucessful scheme depicted here. Of principal significance here is the methods utilized for "visualization" of intermediates which requires not additional steps to prepare. Until these developments, however, work with TTX has been particularly frustrating (see Signficant Negative Results). Our work effort with TTX has utilized 100 mg TTX.

Saxitoxin also has been a difficult toxin with which to work, again primarily due to its scarcity and limited availability even from established sources. STX efforts have been carried out on 40 mg saxitoxin provided by USAMRDC over the past three years. We have attempted to circumvent the source toxin problem in two ways. The first has been to attempt to obtain STX from natural sources including to establish and carry clones of *Gymnodinium catenatum* and produce our own saxitoxin for research, and attempt to obtain sufficient saxitoxin from shellfish (Dr. Sandy Shumway, State of Maine Dept of Marine Resources). The second has been to obtain saxitoxin by total organic synthesis. All of these procurement methods have been carried out under the current contract, but were not originally proposed as tasks.

Saxitoxin derived from shellfish material was minimal and it was conluded that this approach is not sufficiently efficient to warrent further work. Cultured material, on the other hand, does provide highly toxic material, assessed to contain saxitoxin, neosaxitoxin, and GTX 1-4. Our work in this regard has been highly successful and we are currently carrying 320 liter of continuous culture saxitoxin-producing clones. Under separate funding, optimization of toxin purification is continuing.

Saxitoxin synthesis followed the original method of Kishi, and even with the advent of more recent seemingly simpler synthetic schemes, this is still the method of choice. Saxitoxin synthesis is described in this section for the source material is essential for this work, and also because specific toxin site radioactivity can be introduced synthetically during manufacture. The goal was to synthesize racemic d,l saxitoxin, which would then be resolved by chiral column HPLC. Excellent progress has been made in this latter area, and much of the efforts has already been detailed in the mid-term report (Baden, 1991). In particular, our approach has been to prepare and hold sample amounts of each intermediate step for future synthetic comparison, and also in optimizing steps for mass production of saxitoxin (2 g lots). Beginning with the Bunte salt (synthetic scheme on p. 16 of mid-term) and progressing through to the cyanomesylate, we have optimized at over 80% yield (compared with the Kishi original of 40%), and we feel this cannot be improved upon. Specific optimization parameters considered important were absolute dryness of solvents, absence of even tract alcohol in reaction solutions, and obtaining second crops of crystalline material at each step. Principal in our difficulties was obtaining the cyanomesylate (see Significant Negative Results). Significant to state is that once saxitoxin synthesis is complete, the production of neosaxitoxin and many of the natural derivatives can be produced synthetically employing the synthetic saxitoxin and enzymatic conversions. Further work with saxitoxin or any of its derivatives requires amply supplies of starting material.

Tritiated saxitoxin syntheses began with saxitoxin itself as supplied by the US Army. Saxitoxin was decarbamylated as described in detail in the mid-term report. Decarbamylation yields are about 40-60%. Presently, decarbamylated saxitoxin is being prepared as the acid chloride in preparation for reduction to produce tritiated decarbamoyl saxitoxin. This material will be recarbmylated to saxitoxin.

Significant Negative Results

Brevetoxin, Derivatives, and Site Characterization - Our brevetoxin work has progressed very well in terms of each of the derivatives we needed for research and deliverables, and yields have been optimal. Two areas posed some problems, and are worth mentioning. (1) enzyme synthesized high specific activity brevetoxin (approaching 50 Ci/mmole) has not been successful. Although brevetoxn is a substrate for alcohol dehydrogenase reduction employing NAD^{3}H could not produce a specific activity higher than about 20

Ci/mmole. Longer synthetic schemes to produce the acid chloride of PbTx-2, followed by reduction theoretically will yield brevetoxin at specific activities of 20 Ci/mmole (PbTx-3), or 30 Ci/mmole (PbTx-9). Additional work we are exploring involves the methylation of the C-37 hydroxyl of brevetoxin with C^3H_3I to produce tritiated methoxy brevetoxin. Radioactive reagents are available at 60 Ci/mmole and successful derivatization can results in tritiated methoxy-forms of <u>all</u> brevetoxins to allow for measurement of relative binding constants. Site characterization beyond what we have already accomplished, taking the brevetoxin binding site down to domain IV of the α-subunit of the VSSC, requires hotter material for unequivocal optimal work. We had expected to progress to sequencing of the site by this time. We anticipate the brevetoxin binding site characterization will be complete by 1994-1995 and will be the first microspecific toxin binding site described.

Photoaffinity Probes Labeled with Tritiated p-azidobenzoic acid - Two separate custom preparations of ring-tritiated p-aminobenzoic acid were contracted through Amersham Corporation Custom Syntheses. Each preparation has specific activities in excess of 10 Ci/mmole and were individually diazotized to yield p-azidobenzoic acid. In each toxin case, derivatives could be made, and they were calculated to have the expected spcific activities. However, the stability of the derived materials was quite low, and in fact the reagent tritiated p-aminobenzoic acid continued to decompose as obtained by synthesis. Such a derivative is not useful and likely generates tritiated volatile material upon decomposition. This result precludes any further work with this potential derivative as a probe. We conclude that, in the absence of tritiated photoaffinity azides, that radioactive photoaffinity probes will be of the type that incorporates radioactive toxin.

Saxitoxin Synthesis - Saxitoxin synthesis is progressing more slowly than originally anticipated, but the goal far outweighs the time necessary to complete the work. Having sufficient saxitoxin for construction of probes will circumvent many of our current problems. With respect to the synthesis, one step occupied approximately 7 months of our time, but in the end was successful and gave us the exceptional 90% yield. This step, the synthesis of the cyanomesylate, could not be made to reactin the manner described by Kishi. The principal difficulty was the rearrangement of the cyano function to the isocyanide. Repeated attempts to circumvent the rearrangement failed and our optimum yield was 5%. An alternative approach was developed in the laboratory, one which is technically simpler and gave the stated 90% yield. The new appraoch involves the use of bromoacetaldehyde dimethyl acetal, in the presence of butyllithium (instead of diisopropyamide). Improved yields of the cyanomesylate were obtained immediately with now no rearrangement of the cyano. This is the pivotal step in synthesis.

Batrachotoxin - Batrachotoxin was listed as a possibility for derivatives of the type described in this report, and attempts were made to secure 100 mg for synthetic derivative work. Inquiries to Dr. John Daly, Head of Bioorganic Chemistry at NIH, there was not sufficient material for this effort. As an alternative, we utilized other site two toxins veratridine and aconitine, and produced derivatives of the ypes described, both for use as deliverables, and for our internal research use.

CONCLUSIONS

Implications of Completed Work

Natural toxins and their derivatives continue to be important ligands for the investigation of how nerves and muscles work. As intact unaltered materials, each individual class of toxin has been important in describing and probing one specific aspect of neuromuscular transmission. Principal amongst the natural toxins are the marine natural ion channel toxins. These materials have been and continue to be, the most important tools of the neurotoxicologist interested in the structure and function of ion channels. In a specific sense, the toxins interact to provide microspecific allosteric modluation in normal ion channel polypeptides to either activate or inactivate the channel, and also provide derivatives, which by the nature of the toxins themselves, can be delivered to areas of nerve and muscle with nearly quantitative efficiency.

The brevetoxin work continues to succeed admirably, partly based on the virtual unlimited supply of brevetoxin produced by our laboratory. The optimization and development of individual toxin derivative tools is largely because of the good supply of toxin. Site characterization of the brevetoxin receptor to the Domain IV polypeptide region of the α-subunit is unique---brevetoxin are the only toxin currently known to bind to one domain of the VSSC (and not spanning more than one domain like the scoprion toxins or batrachotoxin). This is extremely importnat, then, for it implies that brevetoxn binding to its site on the VSSC can all by itself induce an open state to the channel (its physiological effect). A second lower affinity binding site has been described which has its half-maximal binding constant at concentrations which modulate neurotoxins binding at other sites on the channel. Is this an allosteric modulation site?

At either site, are binding and activity sequential? Are there binding antagonists available or theoretically possible which could act as therapeutic modulators of toxicity? Together with our synthetic, biochemical, and receptor binding pharmacology work, we have begun a theoretical approach to toxin action using computer modeling. While anticipating the usefullness of this approach, expectations have been far exceeded and using modeling routines, we expect a full molecular description of how toxins act with membranes to be forthcoming. The approach has predicted polyether toxin potency based on flexibility and hydrophobicity, and has allowed for development of new toxin tools to further refine the models. Site 5 associated with the α subunit of the voltage-sensitive soidum channel is near full description.

In itself, this is a monumental task and has taken many years of intense research just for the brevetoxins. Further work is forthcoming in this area through collaboration set up with William Catterall in Seattle and with the University of Miami NIEHS Marine and Freshwater Biomedical Sciences Center toxin focus. A principal goal whose implications are immense is the description of the relative binding locales and domains for "site 1", "site 2", relative to "site 5". The efforts require the appropriate toxin tools, which we are continuing to develop and refine, and requires essentially unlimited quantities of toxin for use, hence saxitoxin synthesis.

Future Work Recommendations

(1) Continue brevetoxin work, for its complete description will provide essential information on how and why sodium channels activate. The combination of biochemical, pharmacological, modeling, toxin derivatization including radioactive forms appear as essential components to answer the question. Emphasis should be on hotter radioactive probes like methylated or multiple reductive forms, new appropriate derivatives, investigations of individual binding and activity domains on the toxin and on sequencing the brevetoxin-specific site;

(2) Continue the saxitoxin derivatization work, for the total description of its toxicity will provide information on how and why sodium channels may be shut off---in an antagosistic manner to brevetoxin interaction. Currently, there are no derivative saxitoxin probes available for research outside of tritiated (exchangeable) saxitoxin. Saxitoxin probes, and saxitoxin itself, are essential in research grade quality and quanitity. Emphasis should be on toxin supply by synthesis and culture, and on photoaffinity and other radioactive saxitoxin derivatives which do not exchange (lose) their tritium during research protocols. ^{14}C saxitoxin would appear appropriate for pharmacokinetic metabolism studies;

(3) Continue tetrodotoxin work, for it provides a tool by which sodium channels may be probed in a manner slightly different from saxitoxin (a Site 1 sub-type over lap) in activity and potency. Tetrodotoxin has the added advantage of commercial availability so that once appropriate derivatives are produced, publication of the methods will allow other investigators to follow protocols;

(4) Abandon ring-labeled p-azidobenzoic acid photoaffinity probes, for they are unacceptably unstable;

(5) Abandon enzyme-labeled tritiated brevetoxin syntheses and concentrate on other organic methods as described in (1) above;

(6) Provide amounts of all derivatives and/or intermediates of synthsis for use in intramural laboratories of USAMRDC and their contractees.

PUBLICATIONS AND MEETING ABSTRACTS ARISING FROM SUPPORT

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Baden, D.G., Trainer, V.L., Edwards, R.A., and Mende, T.J. The Binding of Red Tide Brevetoxin PbTx-3 to Rat Brain Synaptosomes: Influence of Membrane Potential, Derivative Brevetoxins, and Site Multiplicity. Brain Research. in press

Trainer, V.L., Thomsen, W.J., Catterall, W.A., Baden, D.G. Photoaffinity Labeling of the Brevetoxin Receptor on Sodium Channels in Rat Brain Synaptosomes. Molecular Pharmacology. in press

Trainer, V.L., Baden, D.G. An Enzyme-Immunoassay for the Detection of Florida Red Tide Brevetoxins. Toxicon. in press

Baden, D.G., Mende, T.J., King, R.W., and Schulman, L. Brevetoxin PbTx-9, a new Toxin Isolated from Cultures of Ptychodiscus brevis. Tetrahedron Letters. submitted.

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PERSONNEL COVERED BY CONTRACT SUPPORT

Name	Role in Project	Covered Period	$ Amount
Daniel G. Baden	P.I. (50% effort)	10/88 - 07/91	295,784
Thomas J. Mende	Co-P.I. (59% effort)	08/88 - 07/91	123,589
Edward Hyde	Research Associate (100%)	07/89 - 05/91	52,407
Lloyd Schulman	Research Technician (100%)	09/88 - 07/91	60,621
Susan Matsuura	Research Technician (100%)	01/89 - 02/89	4,271
Jesus Delgado	Research Technician (100%)	10/89 - 12/89	1,747
Rich Edwards	Graduate Res. Asst (50%)	12/89 - 07/91	24,550
Vera Trainer	Graduate Res. Asst. (50%)	12/89 - 01/90	2,350
Masao Kinoshita	Res. Assoc./Grad. Res. Asst	09/88 - 07/91	64,334
Sean Chemowith	College Work Study	02/90 - 05/90	232
Michell Zetwo	College Work Study	08/89 - 08/90	2,397

GRADUATE DEGREES RESULTING FROM CONTRACT SUPPORT

Vera Lynn Trainer, Ph.D. "Localization of the Brevetoxin Binding Site on the Voltage-Sensitive Sodium Channel". Conferred December 1990, University of Miami Department of Biochemistry and Molecular Biology, 120 pp. *External Examiner: Dr. John W. Daly, NIH.*

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Algal Toxins in Seafood and Drinking Water. (I.R. Falconer, Ed), in press.

Brain Research. in Press.

Molecular Pharmacology. in Press.

Toxicon. in press.

Tetrahedron Letter. submitted.